Using Chemical Approaches to Understand RNA Structure and Function in Biology

Guest Editors: Dmitry A. Stetsenko, and Arthur van Aerschot
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# Editorial Board

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Interest of the research community in the aspects of chemical biology of RNA has increased vastly over the last twenty years, primarily due to the discovery of RNAi, our deepened understanding of the role of miRNA in the subtle regulation of vital cellular processes, and the realization of the fact that the RNA world—the realm where RNA plays the key parties as a self-replicating molecule and a universal catalyst—is still pretty much with us today as the ribosomal RNA performs its catalytic solo in the formation of the peptide bond in the ribosome. To answer the needs of Biology, Chemistry had, in turn, multiplied and perfected its approaches to study the molecular mechanisms underlying RNA functions in living systems. So, the idea behind this special issue is to show our readership a screenshot of what could be, and has been, achieved recently by applying chemical methods to solve the problems of RNA biology. Ten articles have been carefully selected out of the bunch of those submitted to provide, we believe, a balanced view of different facets of RNA structure and function and, also, of the array of chemical tools to enable us to peek into them.

The issue is a mix of reviews and research papers and thus falls into two parts. We have purposefully incorporated a number of authoritative overviews to paint a wider background of RNA functioning in the postgenomic era. A Herculean task of the identification of minor nucleotides in RNA and the use of specific chemical reagents for its accomplishment are reviewed in the contribution from a group of French researchers led by Professor Y. Motorin. A review by A. Serva et al. of the application of high-throughput functional analysis for elucidation of the multiple biological roles of miRNAs. Here we cross the boundary into the second half of our special issue composed from research papers, and the one that comes next by K. Terazawa et al. opens the RNA interference section with synthetic short hairpin RNAs (shRNAs) as potent RNAi inducers. Another article, commissioned by researchers from the Iberian Peninsula R. Eritja and coworkers, deals with branched RNA architectures for RNAi.

Finally, a paper of the issue done by M. A. Zenkova et al. focuses on an applied aspect of RNA technology, namely, the development of sequence-specific artificial ribonucleases
incorporating multiple imidazole residues that perform similar function to their counterparts in natural RNA-cleaving enzymes.

In conclusion, the issue attempts to present a selection of reviews and research papers, which are designed to illustrate how by using various implements and gadgets from the ever-growing arsenal of Chemistry one can hopefully gain a better understanding of what is happening when RNA molecules interact with their manifold targets and perform their plethora of functions. It must be added that this is by no means a comprehensive picture but rather a momentary screenshot of a dynamic process of harnessing the power of Chemistry to serve the ends of Biology. Sapienti sat.

Dmitry A. Stetsenko
Arthur van Aerschot
Chemical Approaches for Structure and Function of RNA in Postgenomic Era

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In the study of cellular RNA chemistry, a major thrust of research focused upon sequence determinations for decades. Structures of snRNAs (4.5S RNA I (Alu), U1, U2, U3, U4, U5, and U6) were determined at Baylor College of Medicine, Houston, Tex, in an earlier time of pregenomic era. They show novel modifications including base methylation, sugar methylation, 5′-cap structures (types 0–III) and sequence heterogeneity. This work offered an exciting problem of posttranscriptional modification and underwent numerous significant advances through technological revolutions during pregenomic, genomic, and postgenomic eras. Presently, snRNA research is making progresses involved in enzymology of snRNA modifications, molecular evolution, mechanism of spliceosome assembly, chemical mechanism of intron removal, high-order structure of snRNA in spliceosome, and pathology of splicing. These works are destined to reach final pathway of work “Function and Structure of Spliceosome” in addition to exciting new exploitation of other noncoding RNAs in all aspects of regulatory functions.

1. Introduction

A key element in the study of cellular RNA metabolism is the molecular characterization of RNA. This characterization requires accurate determination of the RNA sequence. It is imperative to understand how RNA structure complements the functional definition of RNA. Cellular RNAs are posttranscriptionally modified at various points in the primary RNA transcript as well as processed. In cellular RNA metabolisms, RNA maturation is performed through various structural alterations that include chemical modifications of constituent components. A most representative modification is observed in chain shortening, rearrangements by transfer of phosphodiester linkages involved in splicing mechanisms (pre-mRNA), deletions (pre-rRNA), and transsplicing (trypanosomal mRNA). Another is chain expansion demonstrated by modifications observed on polyadenylation, U-addition at 3′ ends, 5′-cap formation at 5′ ends, and insertions within trypanosome RNA. Other examples of modifications are base modifications, such as deaminations, methylations, hypermodifications, and ribose methylations.

The most modified RNAs are tRNAs containing approximately 2–22 modified nucleotides per molecule of ~75 nucleotide length, and there have been more than 130 different signature modified nucleotides reported [1]. The discovery of snRNA and m32,2,2,7G caps occurred within the last 50 years. They also contain their own specific modified nucleotides such as Ψ, m6A, m2G, and 2′-O-methylated nucleotides (Table 1).

The next class is the ribosomal RNAs which contain 204–209 modified nucleotides within 18S (1,869 nt) + 28S (5,035 nt) RNA in eukaryotes. The mRNAs contain the least modified nucleotides, with the exception of the 5′ end cap structure and occasional m6A in the molecule.

In ensuing years, massive scale DNA sequencing was advanced to accommodate the “Human Genome Project.” Two groups published the genomic map where the coding genes were cataloged. It was conservatively estimated that there are 25,000 genes and 50,000 proteomes involved in cell metabolism. It was also envisioned that processing mechanisms could be discerned by comparing the genomic structure with the RNA sequence determined using cDNA methods. Based
Table 1: Signature sequences and modifications of major snRNAs. The 5' cap and 3' nucleosides, base modified nucleosides, and alkali resistant oligonucleotides were determined by many methods described in the text. The table provides a summary of individual RNA characteristics of rat Novikoff hepatoma cells.

<table>
<thead>
<tr>
<th>RNA</th>
<th>Subspecies</th>
<th>Localization</th>
<th>5' End</th>
<th>3' End</th>
<th>Modified Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II (U6)</td>
<td>Extranucleolar nuclei</td>
<td>mpppG</td>
<td>U-OH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>Extranucleolar nuclei</td>
<td>pA</td>
<td>Um</td>
<td></td>
</tr>
<tr>
<td>5S RNA</td>
<td>I</td>
<td>Nucleoli, nuclei and cytoplasm</td>
<td>pppG</td>
<td>U-OH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Nucleoli, nuclei and cytoplasm</td>
<td>pppG</td>
<td>U-OH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III (U5)</td>
<td>Extranucleolar nuclei</td>
<td>m3,2,7GpppAmUmAC</td>
<td>U-OH</td>
<td>UmU, GmC, 2Ψ</td>
</tr>
<tr>
<td>U1 RNA</td>
<td>U1a (5.8S RNA)</td>
<td>Nucleoli and cytoplasm</td>
<td>pC, pG</td>
<td>U-OH</td>
<td>UmG, GmC, Ψ</td>
</tr>
<tr>
<td></td>
<td>U1b, U1c</td>
<td>Extranucleolar nuclei</td>
<td>m3,2,7GpppAmUmAC</td>
<td>U-OH</td>
<td>GmC, 2Ψ</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U2 RNA</td>
<td></td>
<td>Extranucleolar nuclei</td>
<td>m3,2,7GpppAmUmC</td>
<td>C-OH, A-OH</td>
<td>GmGmC, GmG, GmA, mαAmG, CmΨ, UmA, CmU, 13Ψ, (mαA, m2G)</td>
</tr>
<tr>
<td>U3 RNA</td>
<td>U3a, U3b, U3c, U3d</td>
<td>Nucleoli</td>
<td>m3,2,7GpppAmA(m)AG</td>
<td>A-OH, U-OH, C-OH</td>
<td>2Ψ</td>
</tr>
</tbody>
</table>

on the ever-increasing number of RNA sequences, it was determined that most coding RNAs mature as a result of alternative splicing. Aberrant splicing is attributed to point mutations in the genetic code and splicing code [2]. It is noted that RNA sequencing can aid the determination of the molecular pathogenesis of diseases.

2. Historical Venture of RNA Research

Detailed nucleic acid chemistry began with discoveries of the DNA helix by Watson and Crick [3] and DNA polymerase by Lehman et al. [4, 5]. With DNA being the genetic material providing a blue print for living creatures, it moved genomic era thinking away from the earlier notion that protein, carbohydrate, and lipid were the only essences of living things.

DNA is there to provide information needed to build the cells, tissues, organs, and whole individuals. It took a long time to move from the histochemical presentation of DNA in the nucleus and RNA in the nucleolus and cytoplasm [14] to the isolation of nucleoli, nuclei, mitochondria and ribosomes, facilitating the elucidation of their components, their structures, and their functions. Even within the same species, no two individuals are identical. Disarray in DNA structure can determine whether one is healthy or diseased. In the quest to conquer cancer, differences in cellular morphology and uncontrolled growth became and remain a major research consideration when one compares normal cells with cancerous cells and tissues. Cancer cells with pleomorphic, hypertrophic nuclear, and nucleolar morphology remain a useful pathological criterion for a cancer diagnosis. The information within genes is transferred to RNA and then to proteins made on ribosomes that define a cell phenotype. The fractionation of cells into various components includes nucleoli, nuclei (Figure 1), ribosomes, mitochondria, cytosol and others.

The main interest among these compartmental components was the RNA. The RNA has its own exclusive properties which are not found in DNA.

The discovery of RNA polymerase I in the nucleoli [31] is the landmark of RNA research in these cellular compartments. It was not until 1968, with the introduction of gel electrophoresis into RNA research [32], that subspecies of 4–8S RNAs could be separated from high-molecular-weight RNAs (>18S RNA). Until then, the 4–8S RNAs were considered as tRNAs and their precursors. Different from the prokaryotic cells, eukaryotic cells were shown to have a variety of small RNAs in their nuclei (Figure 2). These RNAs used to be called LMWN RNA (low-molecular weight nuclear RNA) and now the name is unified as snRNA (small nuclear RNA).

These include U1 RNA, U2 RNA, U3 RNA, (named as such because these RNAs contain a high proportion of uridylic acid), 5S RNA III (U5 RNA), 4.5S RNA I (Alu RNA), 4.5S RNA II (U6), and 4.5S RNA III. All of these snRNA species and many more have been sequenced and their functions elucidated in pre-rRNA processing [33] and pre-mRNA splicing [34, 35].
Nuclear and nucleolar morphology

(A) Normal liver

(B) Regenerating liver

(C) Hepatocellular carcinoma

Isolated nucleoli

(A) Normal rat liver nucleoli

(B) Walker 256 carcinoma nucleoli

EM picture of isolated rat liver nuclei

EM picture of isolated rat liver nucleoli

**Figure 1**: Nuclear and nucleolar morphology. Normal rat liver nuclei have 1–6 round nucleoli which are less than 2 μm in diameter. In regenerating liver, cells contain enlarged nucleoli. In tumor cells (hepatocellular carcinoma), the nucleoli are not only enlarged but also they become pleomorphic in morphology. Nuclei were isolated by homogenization in 2.3–2.4 M sucrose containing 3.3 mM CaCl₂. Nuclei were sonicated in 0.34 M sucrose and layered on 0.88 M sucrose for purification by centrifugation. Isolated nuclei and nucleoli had high purity, and morphologies were well preserved [6].
The most interesting discoveries in the midst of sequencing were the very unusual trimethylguanosine cap structure in U1 RNA (m32,2,7GpppAmUmAC), U2 RNA (m32,2,7GpppAmUmC), U3 RNA (m32,2,7GpppAmA(m)AGC), and 5S RNA III (U5 RNA) (m32,2,7GpppAmUmAC) [36]. Afterwards, myriads of cap structures in viral RNA and mRNA were discovered [37].

The history of RNA sequence work has occurred in three eras. The pre-genomic era was devoted to the small RNAs and commenced with the sequence of large RNAs as technology developed for cDNA synthesis, amplification, cloning, and sequencing. The DNA technology was explosive and paved the way toward establishment of sequence technology not only for RNA and cDNA but also for genomic DNA.

In addition to sequence study, the secondary and tertiary structures have also been determined. A representative study was the crystallographic study of RNA-protein interactions. For example, the most well-worked-out motif is RRM (RNA recognition motif) which is most abundant in hnRNP [40] and splicing factors [41]. The summary of characteristics of RRM is in Table 2.

It has been known for a long time that pre-mRNA (hnRNA) is cotranscriptionally assembled into beads on a string consisting of 30–50S (20–30 nm) particles [42]. The
Figure 3: The supraspliceosome model from the article by Sperling et al. [8]. (a) It was stated that pre-mRNA which is not being processed is folded and protected within the native spliceosome. (b) With different staining protocol, it was possible to visualize the RNA strands and loops emanating from the supraspliceosome. These complexes were found to contain hnRNP proteins (personal communication).

RNP (hnRNP) has usually 48 hnRNP proteins and ~700–800 nucleotide long RNA string [43]. More recently, most hnRNP proteins have been found to have 1-2 RRM motifs for RNA binding. From these characteristics, the primary RNA transcripts have been folded from the 5' end with the following rules: a minimum of 3 nucleotides in the loop and a minimum of 3 base pairs at the stem. According to stacking and loop energy rules, two nucleotide loops cannot exist. The number of base pairs needed for stabilization with the most stable stacking energies by CCC/GGG or GGG/CCC is 3 base pairs with −9.8 kcal and the highest loop destabilizing energy is +8.4 kcal [44]. In addition, protein binding to RNA has been shown to have −ΔG ≈ 10–13 Kcal/mol [45] which can overcome the loop destabilizing energies of any size. With this rule, folding the hnRNA in GC, AU, and GU pairings was carried out as the RNA was transcribed, extending contiguous base pairing until it comes to a base pair mismatches. Accordingly, small simple RNA hairpins have been constructed with the aid of a computer [46] from the 5' end (transcription start sites). Consensus patterns for folding characteristics have been observed (Table 3).

The transcripts form one stem loop for every 15–18 nucleotides which is consistent with ~15–17 nucleotides per hnRNP protein (700–800 nucleotides per 48 hnRNPs in one hnRNP particle) reported earlier [43]. The thermodynamics of RNA folding was consistent with the order of splicing in ovomucoid pre-mRNA [47]. From the point of view that supraspliceosomes contain hnRNP proteins (personal communication), it may be that this cotranscriptional formation of hnRNP string particles [47–49] may contribute to a role in the formation of supraspliceosomal RNP (Figure 3) [8].

The postgenomic era is the present day era or the second generation genome era. With the recent discovery that there is a paradox [50, 51] in the cellular transcript number, which is 2-3-fold in excess and that 50% of the cellular transcripts are ncRNAs, the second generation genomic era is in the process of resequencing the genome for ncRNAs. It is anticipated that there will be a revision in the first generation genomic picture. In this era, work is proceeding that will probe and dissect the RNA metabolism in which aberrant processing should be elucidated by RNA sequencing. To dissect the molecular pathology of RNA metabolism, it is also necessary to study higher-order structures based on the sequence studies involved in the assembly of macromolecular machinery. It is natural to hope that therapeutic interventions will be discovered that can correct errors in the genetic code and its product splicing.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>nt/loop</th>
<th>nt/stem</th>
<th>nt in spacer</th>
<th>Frequency</th>
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<tr>
<td>(1) Insulin</td>
<td>4.6</td>
<td>7.4</td>
<td>3.5</td>
<td>15.5</td>
</tr>
<tr>
<td>(2) HDHGT</td>
<td>5.8</td>
<td>7.0</td>
<td>4.9</td>
<td>17.6</td>
</tr>
<tr>
<td>(3) FMR1</td>
<td>5.0</td>
<td>6.8</td>
<td>3.4</td>
<td>15.3</td>
</tr>
<tr>
<td>(4) Ovomucoid</td>
<td>5.6</td>
<td>7.0</td>
<td>3.7</td>
<td>16.0</td>
</tr>
</tbody>
</table>
Table 4: Paradoxical characteristics of ncRNAs in humans and mice [50, 51]. The excessive number of transcripts than anticipated for 25,000 genes indicates that the ncRNAs which were not detected due to scarce abundance have been detected by more sensitive methods. Some of these characteristics are summarized.

<table>
<thead>
<tr>
<th>Gene Number</th>
<th>Human</th>
<th>Mouse</th>
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<tbody>
<tr>
<td>Transcripts</td>
<td>75,000, 84,000 or 140,000 (cDNA identified)</td>
<td>181,000</td>
</tr>
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<table>
<thead>
<tr>
<th>Population</th>
<th>50% Poly-A RNAs (of 16% genome)</th>
<th>50% transcripts (of 62% genome)</th>
</tr>
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<tr>
<td>Intron</td>
<td>30% genome</td>
<td>(35% from antisense strand)</td>
</tr>
<tr>
<td>Processing</td>
<td>Polyadenylation, 5’ cap, splicing, nucleotide modification</td>
<td></td>
</tr>
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</table>

<table>
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<tr>
<th>Transcripts from</th>
<th>Intergenic, Intronic regions and antisense strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short ncRNAs</td>
<td>miRNA, siRNA (tasiRNA, natsiRNA), piRNA, rasiRNA (pitRNA), PARs (PROMTs, PASRs, TSSa-RNAs, tiRNAs), MSY-RNA, snoRNA, sdRNA, moRNA, tel-sRNA, crasiRNA, hsRNA, scarRNAs, AluRNA, YRNA, tRNA-derived RNAs</td>
</tr>
<tr>
<td>Long ncRNA (lncRNA)</td>
<td>Cancers, disorders in skin, heart, brain, cerebellum, and so forth. TR/TERC, NEAT RNA (NEAT1v-1, NEAT1v-2, NEAT2/MALAT1), PINC RNA, DD3/PCA3, PCGEM1, SPRY4-1T1, xiRNAs (Xist RNA, Tsix RNA, RepA RNA), AIR, H19, KCNQ1ot1, HOTAIR, BORG, CTN RNA, ANRIL RNA, LINE, CSR RNA, satellite DNA transcripts and so forth</td>
</tr>
</tbody>
</table>

| Function | Regulatory function in all aspects of metabolism [52] |

The RNAs have been classified according to the following diverse basis of criteria:

(i) cell biology: cell types, subcellular origins,
(ii) molecular weight: high molecular weight (HMW) and low molecular weight (LMW/small),
(iii) S value: 5S rRNA, 7S RNA, 18S RNA, and others,
(iv) linearity: linear, cyclized, and branched (Y shaped),
(v) metabolism: precursor, processed intermediates, and mature,
(vi) standard: hnRNA, rRNA, mRNA, tRNA, and ncRNA (snRNA, snoRNA, miRNA, and others as in Table 4).

3. Preparation of RNA from Isolated Subcellular Compartments

RNA can be extracted from purified nucleoli, nuclei, ribosomes, mitochondria, and cytosol by the SDS-phenol procedure. The procedure involves the suspension of organelles in 0.3–0.5% SDS (sodium dodecyl sulfate), 0.14 M NaCl, and 0.05 M sodium acetate buffer at pH 5.0 and deproteinization by phenol containing 0.1% 8-hydroxyquinoline at 65°C [53]. The extracted RNA is precipitated with 2–2.5 volumes of ethanol containing 2% potassium acetate. The RNA is washed by ethanol and dissolved in appropriate buffer for the analysis. The DNA and protein contaminations are less than 3% by weight. The purified RNA is separated into individual RNA species using sucrose density gradient centrifugation, gel electrophoresis, and column chromatography [38].

4. Structure Determination

4.1. Structural Characteristics of Various RNAs Bearing Signature Sequences and Modifications. The RNA is composed of basic 4 nucleosides of guanosine, adenosine, uridine, and cytidine linked by 5’-3’ phosphodiester bonds between two ribose moieties. In addition, some of these nucleotides are modified in base as well as in ribose moieties and contain unusual pyrophosphate bonds at their 5’ ends and 2’ O-methylated 3’ end.

Mature RNAs are synthesized in the nuclei and directed by the posttranscriptional processing machineries. Because of these specific modifications, there is a general consensus on the presence of specific signature sequences and modifications for the identity of RNA classes. Based on extensive sequence work, it is possible to classify RNAs according to structural modifications. Figure 4 provides an outline for characteristics of RNA, and its modifications and brief examples are given in Table 5.

4.2. General Scheme of RNA Sequencing. The very first RNA sequence was obtained from the work of yeast alanine tRNA in 1965 [54]. In this work, the prerequisites for RNA sequence work were developed and described. Since then, it is a fundamental approach to establish oligonucleotide catalogs using specific RNases. One set is the catalog of T1 oligonucleotides produced by RNase T1. The other is the catalog of oligonucleotides produced by RNase A. The analytical method was based on UV spectral absorption in the earlier years. Subsequently, since 1970, isotopic labeling methods were widely used which are 1,000-fold more sensitive. Furthermore, many other improvements in RNA
Deletion
Splicing
intron 5
- end
Cleave off
Insertion
Editing
deamination
Ribo
e methylation
- end

m\textsubscript{2,2,7}G-cap
m\textsubscript{2,7}G-cap
m\textsubscript{7}G-cap
m\textsubscript{γ}G-cap
pppN, pN,
Base modifications by moieties of
alkane, alkene, amino acids, lipids, carbohydrates
ring formation, sulfur, and selenium

Figure 4: Summary of RNA modifications. Cotranscriptional and posttranscriptional RNA modifications are summarized.

Table 5: Examples of modification. All RNA species including high- and low-molecular-weight RNAs have their own signature sequences and modifications.

<table>
<thead>
<tr>
<th>RNA</th>
<th>Sequence and signature modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>hnRNAs</td>
<td>m\textsubscript{7}G cap, m\textsubscript{2}A, Poly-A, splicing codes</td>
</tr>
<tr>
<td>(Exons + Introns)</td>
<td></td>
</tr>
<tr>
<td>mRNAs</td>
<td>m\textsubscript{7}G cap, m\textsubscript{2}A, Poly-A</td>
</tr>
<tr>
<td>(mainly exons)</td>
<td></td>
</tr>
<tr>
<td>tRNAs</td>
<td>m\textsubscript{3,2,7}G cap, myG cap</td>
</tr>
<tr>
<td>45S pre-rRNA</td>
<td>Repeated U sequences at 5'-end spacers</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>NmNmNmN, NmNmN</td>
</tr>
<tr>
<td>28S rRNA</td>
<td>NmNmNmN, NmNmN</td>
</tr>
<tr>
<td>snRNAs, snoRNAs</td>
<td>Types I, II, and III caps by ribose methylations</td>
</tr>
<tr>
<td>mRNA</td>
<td>m\textsubscript{7}Gpppm\textsubscript{3,6}AmpAmpCmpm\textsubscript{3}UmpAp</td>
</tr>
<tr>
<td>(Trypanosome)</td>
<td>Insertion of repeated U sequence</td>
</tr>
<tr>
<td></td>
<td>Deletion of U sequence</td>
</tr>
</tbody>
</table>

sequence technique have made it possible to advance the rate of RNA sequence work greatly (Table 6).

Improvement was observed in the following areas: (1) RNA labeling techniques, (2) fractionation procedures (chromatography, electrophoresis, and gel procedures), (3) use of various RNases, (4) contig seeking, and (5) ladder sequence gel analysis. For example, based on labeling at the 5'-end with \textsuperscript{32}P-\gamma-ATP by polynucleotide kinase [56], it has become feasible to read a 150 nucleotide sequence using an endonuclease assisted ladder gel from the 3'-end. Also, based on labeling at the 3'-end with \textsuperscript{32}P-5'-pCp by RNA ligase [57], it has become feasible to read approximately 150 nucleotides from the 5'-end. Together, these enhancements make it readily feasible to sequence RNA with approximately 300 nucleotides. In contrast to success in the sequence work for small RNAs, two challenges remained. One challenge is related to RNA size and the other is concerned with scarce abundance of RNA in the cell. With the discovery of reverse transcriptase, heat stable DNA polymerase, and recombinant technology, it became possible to produce cDNA, amplify, and clone by RT-PCR methods.

With high-efficiency RT-PCR, high-molecular-weight RNA with 10,000 nucleotides in length can be readily sequenced [59]. A remaining shortcoming of this approach is the inability to fully characterize modified nucleotides. However, ability to deal with long chain lengths and scarce abundance outweighs this limitation. cDNA-based methods clearly dominate any RNA sequence work that involves long RNA length or low RNA abundance. Examples are observed in the direct gene isolation for cleavage controlled processing RNAs (Pre-rRNA and rRNA) and cDNA method for pre-mRNA and mRNAs. Therefore, as a result of accumulated methodologies, it becomes common that RNA sequence can be obtained through more than one scheme or type of technique, such as straight chemical approaches [60] or biotechnology-mediated approaches.

4.3. Outlined Steps of Sequence Work. Brief outlines are described for sequencing RNAs. It may be divided into two methods although combined methodology is in fact feasible.

4.3.1. Direct Method of RNA Sequencing

(a) Preliminary Examination of External Glycol Structures. In some cases, a rapid diagnostic examination is required. Most convenient procedures employ the use of specific antibodies against different forms of 5'-cap structure (m\textsubscript{7}G cap or m\textsubscript{3,2,7}G cap) and an oligo-dT column for poly-A affinity chromatography. Alternatively, a \textsuperscript{3}H-derivative method can be useful. The radioactive labeling of terminals was performed using the periodate oxidation method, followed by reduction with \textsuperscript{3}H-borohydride. T\textsubscript{2} RNase digestion and fractionation by paper chromatography reveal the presence of the 3'-terminal and 5'-cap.

(b) Selection of Labeling Methods. RNA can be labeled in vivo (prelabeling) or in vitro (postlabeling).

In vivo labeling is carried out by incubation of living cells in the presence of \textsuperscript{32}P-phosphate in a phosphate-free medium. RNA is uniformly labeled by this method.

In vitro labeling is called postlabeling because it labels the isolated RNA with isotopic agents such as \textsuperscript{32}P-phosphate or \textsuperscript{3}H-borohydride. \textsuperscript{32}P-labeling can be carried out using kinase enzymes. The 5'-labeling is done with \textsuperscript{32}P-ATP by
Table 6: General schemes of RNA sequencing. The direct and indirect methods of RNA sequencings are briefly outlined. The cDNA and DNA pathways are considered indirect methods.

<table>
<thead>
<tr>
<th>Starting material RNA</th>
<th>Starting material RNA</th>
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<tbody>
<tr>
<td>Endonuclease</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Catalogs of oligonucleotides</td>
<td>cDNA library</td>
</tr>
<tr>
<td>Contig seeking</td>
<td>Genomic DNA library</td>
</tr>
<tr>
<td>Large fragments</td>
<td></td>
</tr>
<tr>
<td>Endonuclease assisted sequencing</td>
<td>Screening</td>
</tr>
<tr>
<td>Ladder sequencing gel</td>
<td>Screening</td>
</tr>
<tr>
<td>Sequence</td>
<td>Clone</td>
</tr>
<tr>
<td>+</td>
<td>Clone</td>
</tr>
<tr>
<td>+</td>
<td>Clone</td>
</tr>
<tr>
<td>Modifications</td>
<td></td>
</tr>
</tbody>
</table>

Polynucleotide kinase, that is, provided the 5'-end is free from phosphate. If the 5'-end is blocked by the presence of a 5'-cap structure, the pyrophosphate moiety must be removed by a pyrophosphatase and phosphatase. And then the kinase method can be employed to introduce the tracer. Labeling at the 3'-end is done with [32P]-pCp by RNA ligase. The [3H]-derivative (nucleotide diol) with [3H]-borohydride indicates that the 3'-end is free from phosphate or any other blocking structures. A shortcoming of [32P]-labeling is the short half-life of the isotope which provides a working period of approximately 4 half-lives. The main limitation of the [3H]-labeling method is weak energy of the tritium isotope. This can make the reading of the autoradiograph for a ladder sequencing gel very difficult.

(c) Initial Reading of Sequence by Ladder Sequencing Gel. To obtain the nucleotide sequence of RNA quickly without characterization of modified nucleotides, it is common to use the endonucleases-dependent sequencing technique [61]. Terminal labeled RNA (5'-end or 3'-end) is partially digested with specific endonucleases (T1, U2, A, phys I, and others), and each product is loaded in parallel on a 10–15% denaturing polyacrylamide gel. Note that if crude acrylamide is used, the running temperature of the gel can quickly rise to 60–70°C. Since the mode of cleavage is known, it is possible to discern G (T1), A (U2), U and C (A) and C-resistance (Phys I). It is not uncommon to read an RNA sequence using this method within one day.

(d) Base Composition. There are two technical approaches that can be used to determine RNA base composition (levels of nucleotides or of nucleosides). RNase T2 or alkali (0.3 N KOH) is used to complete hydrolysis. But alkali (0.3 N K/NaOH) is not preferred because
it destroys 7-methyl purines. Prelabeled $^{32}$P-RNA is hydrolyzed, and its products are separated by 2-dimensional paper chromatography followed by autoradiography [62]. Since the standard separation pattern is known, various modified nucleotides are readily identified by comparison [56].

Alternatively, after cold RNA is digested into constituent nucleotides, which are subsequently dephosphorylated by phosphatase, the resulting nucleosides are converted into $^{3}$H-derivatives and separated by thin layer chromatography. The separated nucleosides (including all modified nucleosides except 2'-O-methylated nucleosides) are detected by fluorography and identified based upon a standard migration pattern (Figure 5) [9].

(c) Catalogs of Oligonucleotides. Two types of catalogs are made. One is an RNase T$_1$ catalog, and the other is an RNase A catalog.

To map oligonucleotides, two necessary procedures are essential. The first is to prepare labeled oligonucleotides and the second is to fractionate two-dimensionally.

To obtain labeled oligonucleotides, three approaches are possible.

1. Use of prelabeled $^{32}$P-RNA for specific endonuclease digestion.
2. 5' labeling after enzyme digestion using $^{32}$P-ATP and polynucleotide kinase.
3. 3' labeling after endonuclease digestion and removal of resultant 3'-phosphate by phosphatase. Then the labeled derivatives can be formed by $^{32}$P-5'-pCp and RNA ligase or periodate oxidation followed by $^{3}$H-borohydride reduction.

To Map Oligonucleotides. There are a number of different techniques. However, the most common are a combination of high voltage paper electrophoresis on cellulose acetate at pH 3.5 and high voltage DEAE paper electrophoresis (7% formic acid) or high voltage electrophoresis on cellulose acetate at pH 3.5 followed by DEAE homochromatography at 60–70°C. Another method that can be used is two-dimensional thin layer (PEI) chromatography using two-solvent systems [63]. Detection is performed by autoradiography. It is notable that T1 oligonucleotides from 45S pre-rRNA can be fractionated into approximately 200 spots by homochromatography [64].

To Sequence Oligonucleotides. Several enzymatic digestions can be exploited.

The recovered $^{32}$P-oligonucleotides (prelabeled) are subjected to secondary digestions with RNase U$_2$ for placement of A residues, RNase T$_1$ for G residues, RNase A for U, and C residues plus other endonucleases. Treatment with exonucleases (spleen phosphodiesterase, snake venom phosphodiesterase), and partial digestion with the enzymes above is required to sequence RNA. In each step, nucleotide composition is determined.

To Determine the Sequence of 5'-Labeled $^{32}$P-Oligonucleotides. A mobility shift test can be applied [56]. After partial hydrolysis with snake venom phosphodiesterase the product is fractionated by homochromatography or PEI thin layer chromatography. The mobility shift pattern is produced according to the step-wise loss of each nucleotide from the 3'-end. The resulting pattern can be used to read the sequence of the oligonucleotides.

To Determine the Sequence of $^{3}$H-Oligonucleotides. The procedures used for prelabeled $^{32}$P-oligonucleotides are applicable. Secondary digestion methods and accompanying $^{3}$H-derivative methods for the determination of nucleotide composition can be carried out.

It may be necessary to strengthen the catalog of oligonucleotides. Generally this involves the expansion of the catalog to provide contiguous overlapping sequences. A feasible approach is to produce large fragments (purified on 10–15% denaturing polyacrylamide gel electrophoresis) and identify the overlapping oligonucleotides. Usually a limited fragmentation by a diluted endonuclease at low temperature or water hydrolysis may produce large overlapping fragments [63]. Examination of large fragments, as done above for ladder gel sequencing and catalogs, can often clarify any ambiguity encountered. An excellent example of one hit hydrolysis is observed in the work on tRNA structure [63]. Based on these very same methods, it can be summarized that many small RNAs have been sequenced. These include tRNAs, pre-tRNAs, 4.5S RNA I, 5S rRNA, 5.8S rRNA, snRNAs, snoRNAs, 7S RNA, and some fragments of pre-rRNA, 28S rRNA, and 18S rRNA.
4.3.2. **Indirect Method of RNA Sequencing.** The indirect method of RNA sequencing using cDNA or DNA gene analysis was developed as part of explosive advancements with DNA biotechnology. The direct RNA sequencing method proved useful for the characterization of small RNAs (~100–300 nt). However, sequencing high-molecular-weight (HMW) RNAs proved to be too difficult. Moreover, HMW RNAs that are scarce abundance often do not meet the sample amounts required by the former methods. The search for a solution to this dilemma was successful. One solution involved the isolation of the gene that codes for a specific RNA and the other is to synthesize cDNA which can also be used to isolate a specific RNA gene. Using DNA biotechnology, it proved possible to scale up and solve “The Human Genome Project.” Several genomes have been sequenced, specifically the human (2.9 Gb) and mouse (2.5 Gb) genomes [65–67]. In well equipped laboratories, it is possible to sequence DNA at the rate of $10^6$–$10^7$ nt/day. This technology has been widely commercialized and is currently available as kits for cDNA cloning, sequencing, along with enzymes and equipment that supports automatic sequencing. The principal objective of the genomic approach was to determine the sequences of the coding genes. Vast collections of sequence data were compiled for RNAs, cDNAs, and genomic structures, revealing the base sequences for a number of RNAs. As a result of this work.

(a) Unidentified proteins have been predicted to number 25,243; whereas the known protein number is 15,337.
(b) A majority of mRNA species (95%) mature through alternative splicing mechanisms.
(c) Disease genes are estimated to be 2,577 in number.
(d) Point mutations are 31,250 in number; half of disease-causing mutations are attributed to aberrant splicing (disruption of splicing codes) whereas other forms of mutation include disruption of the genetic code.
(e) Disruption of splicing code occurs at the splice site and enhancer/silencer sites of exonic and intronic sequences.
(f) Pathogenic sequences that occur as a result of splice code mutations (transition and transversion) cause aberrant modifications of a variety of RNAs [68, 69].

Recently, evidence has been accumulating that suggests a need to revise earlier estimates of the number of transcriptional products arising from the genomic information. Paradoxical findings were obtained that contradicted earlier and more conservative estimates of the proteasomes size (50,000), in fact, the cellular transcripts are 2-3 times higher than estimated earlier [50, 51]. Also, 50% of the transcripts were comprised of noncoding RNA, some of which are polyadenylated. This paradoxical manifestation has led to the second generation of genomic work, strictly based on RNA characterization. It is worth emphasizing that this has become the second genomic frontier where a reevaluation of the first genomic work is necessary. The present task is more daunting than the “The first Generation Genome Project.”

The task at hand is to resequence the genome and then categorize and catalogue the ncRNA species by utilizing all available sequence means, including direct sequencing and DNA microarray techniques.

The next step is to construct secondary structures according to enzyme susceptibility and computer-aided base pairing. Interacting proteins will need to be defined by biochemical, NMR, X-ray, and cryo-EM methods.

5. **Reagent and Procedures Required for Sequencing**

5.1. **RNA-Specific Cleavage Reactions (2′-OH Required Reaction)**

1. Mild alkaline hydrolysis (0.3 N KOH) produces 3′ monophosphorylated nucleotides.
2. T1 RNase cleaves phosphodiester bonds after G base producing 3′ GMP at the 3′ ends.
3. RNase A cleaves phosphodiester bonds after pyrimidines (U and C) producing 3′ phosphates at 3′ ends.
4. T2 RNase cleaves all phosphodiester bonds with a preference for A residues, producing 3′ monophosphates.
5. U2 RNase cleaves phosphodiester bonds after A base, producing 3′ monophosphates.

The mechanism catalyzed by alkaline hydrolysis, RNase A, T1 RNase, T2 RNase and U2 RNase involves a $S_{N}2(p)$ mechanism attacking 2′-hydroxyl groups on the adjacent internucleotidic phosphodiester bond to displace the 5′-phosphate linkages, producing 5′ mononucleotide (Figure 6).

5.2. **The Enzymes Cleaving All Phosphodiester Bonds Including 2′-O-Methylated Ribose**

1. P1 RNase: the enzymatic digestion by P1 RNase cleaves all phosphodiester bonds (except pyrophosphate linkages), producing 5′ monophosphorylated nucleotides.
2. The enzymes acting from the ends for sequencing fragments

(a) Snake venom phosphodiesterase (phosphodiesterase I) cleaves phosphodiester bonds, as well as pyrophosphate bonds producing 5′ monophosphorylated nucleotides. It cleaves single-stranded RNA or DNA from the 3′ end in a progressive manner.
(b) Spleen phosphodiesterase (phosphodiesterase II) produces 3′ monophosphorylated nucleotides cleaving from nonphosphorylated 5′ ends of single-stranded RNA or DNA.
requiring enzymatic and alkaline hydrolysis may go through the same path. The intermediary 2′,3′ cyclic nucleotide (cNp or cNMP) is hydrolyzed to a 3′ phosphorylated mononucleotide. Other 2′-OH requiring enzymatic and alkaline hydrolysis may go through the same path.

5.3. Other Enzymes Utilized for Sequencing

(1) Alkaline phosphatase removes phosphate from 3′ and 5′ ribose moieties.

(2) Pyrophosphatase will only cleave pyrophosphate linkages. There are pyrophosphatases from tobacco and potato as well as from Crotalus adamanteus venom type II.

Using varying combinations of fragmentation methods, it becomes possible to obtain fragments that range in size from nucleosides to very large fragments.

5.4. Chemical Modifications Used for Sequencing

5.4.1. CMCT Reaction. Originally reported by Gilham [73], the adduct formation of uridine and guanosine components of RNA with CMCT made uridine residues resistant to RNase A. In addition it has been shown that CMCT reacts with pseudouridine and to a lesser extent with inosine. This reaction takes place on RNase A digestion at U but not at C as well as to from Crotalus adamanteus venom type II.

Using varying combinations of fragmentation methods, it becomes possible to obtain fragments that range in size from nucleosides to very large fragments.

5.4.2. DMS (Dimethylsulfate). This has been used to identify secondary structures as well as for the synthesis of standard m32,2,7G. The properties of DMS modifying adenosine (N1) and cytosine (N3) make modified nucleotides unable to base-pair. For this reason RT-PCR stops one nucleotide before the modified nucleotide before the modified nucleotide enabling the location of a modified nucleotide as well as differentiating the single-stranded from double-stranded regions of RNA. DMS has also been used for synthesis of m32,2,7G from N2,N2-dimethylguanosine. For this synthesis, the reaction has been carried out by the methods of Saponara and Enger [76]. Twenty milligrams of N2,N2-dimethylguanosine were suspended in 400 μL of dimethylacetamide containing 10 μL dimethylsulfate. The mixture was shaken for 15 hours at room temperature and then centrifuged to remove insoluble products. The supernatant was adjusted to pH 8.0 with concentrated ammonia and then placed on a phosphocellulose column (1 × 50 cm) at pH 7.0 (0.001 M ammonium acetate). A linear gradient of 0.001–0.3 M ammonium acetate was used to elute the samples. One major peak of the product (m32,2,7trimethylguanosine) was found between two minor peaks (corresponding to N2,N2-dimethylguanosine and 7-methylguanosine). The product was lyophilized and identified as m32,2,7G by mass spectrometry [12]. The summary of reagent and procedures required for sequencing is provided in Table 7.

The nucleotides or nucleosides obtained can be separated by column chromatography, paper electrophoresis or thin layer chromatography to determine the number of G, A, U, C and modified residues in the fragments or in the molecule. These 4 bases have specific UV spectra and chemical reactivity to identify the nature of the bases in comparison with known standards. The unusual nucleoside, trimethylguanosine, has its specific UV absorption spectra (Figure 8) and mass spectrometric characteristics (Figure 9).

6. The Major snRNA Sequenced

The first nuclear small RNA sequenced was 4.5S RNAI [77] shown in Figure 37. This RNA contains the RNA polymerase III promoter box A and box B like motifs and shows interesting enhancer motif elements resembling the Alu element transcript. The RNA polymerase III promoter areas are underlined and the first nucleotide of the enhancer motif is marked by colored letters. The red color is SF2/ASF (4 motifs), blue color is SC35 (3 motifs), green color is SRp40 (6 motifs), and yellow color is SRp55 (1 motif) (Figure 10(a)). It also exhibits 3′-splice sites marked by [AG] as well as branch sites with the highest score marked by {CACCUAU} (Figure 10(b)). The ESE (exonic splice enhancer), splice sites (Figure 10(c)), and branch sites were examined by ESEfinder 3.0 [13].

In comparison with known Alu elements in the FMR1 gene, the resemblance of 4.5S RNA I in ESE, 5′SS, BS, and 3′SS distribution (Table 8) suggests that 4.5S RNA I is more likely derived from an Alu gene expressed in Novikoff hepatoma cells.

The Alu element has been shown to have many different functions in transcription, splicing, exonization [78], gene insertions (transposons), and DNA replication. It is interesting to observe that the (+) oriented Alu has more
Figure 7: The CMCT reaction of pseudouridine (Ψ) and uridine, and the structure of CMCT [11]. Adducts formed with CMCT on Ψ and U are shown. This adduct formation prevents the cleavage by RNase A at U but not at C. The mild alkaline treatment of reaction products destroys the U but not the Ψ. These differences were utilized to locate the position of Ψ by reverse transcriptase.

7. Nucleotide Composition and Modified Nucleotides in snRNAs

The compositional analyses were carried out by UV analysis as well as isotope labeling analysis. For example, UV analysis required ~10 mg of U2 RNA.

7.1. RNA Terminal Labeling with [3H]-KBH₄. The purified nuclear RNAs were separated by sucrose gradient centrifugation which separates 4–8S RNA, 18S RNA, 28S RNA, 35S RNA, and 45S RNA isolated from nuclei of rat liver, Walker tumor, or Novikoff hepatoma cells. As an initial step for the structural characterization, 3′ end nucleosides were labeled by the procedure of sodium periodate (NaIO₄) oxidation and potassium borohydride ([3H]-KBH₄) reduction.

5′ splice sites and the (−) oriented Alu has more 3′ splice sites. It may suggest that exonization may occur from the 5′ side of (+) Alu elements and 3′ side of (−) Alu elements. The SRP RNA (7SL RNA) has Alu elements in its sequence [79]. Whether the Alu is derived from 7SL or Alu is exonized to 7SL is not clear. Subsequently, other snRNAs have been sequenced.

The sequences of the capped snRNAs are described in Figure 11. The pivotal sequences needed for functions are marked by colors.

In the course of any sequence work, there are always challenges in resolving unknown structures at the 5′ end portions which contain the 5′-cap structure and various modified nucleotides. The experimental steps required to discern this complicated region are described.
Table 7: Reagents and procedures required for sequencing.

<table>
<thead>
<tr>
<th>Detection of external modification</th>
<th>Oligo-dT column</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Borate column</td>
</tr>
<tr>
<td></td>
<td>Antibodies against m7G, m3,2,7G, and others</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Labeling</th>
<th>Prelabeling (in vivo) with [32P]-phosphate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Postlabeling (in vitro)</td>
</tr>
<tr>
<td></td>
<td>5' [32P] labeling</td>
</tr>
<tr>
<td></td>
<td>3' [32P] labeling</td>
</tr>
<tr>
<td></td>
<td>3' and 5' [3H]-derivative labeling</td>
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</tbody>
</table>

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<thead>
<tr>
<th>Enzymes</th>
<th>RNase T1(Gp↓N), RNase A(Up↓N or Cp↓N), RNase U2(Ap↓N), PhysI(C-resistance), RNase T2(Np↓N), RNase P1(pNm↓pN, pN↓pN), Debranching enzyme N2↓5pNp3↓pNp</th>
</tr>
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<tr>
<td>Endonucleases</td>
<td>Spleen phosphodiesterase (Np↓Np↓Np)</td>
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<td>Exonuclease</td>
<td>Snake venom phosphodiesterase (N↓pN↓pN)</td>
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<table>
<thead>
<tr>
<th>Phosphatase</th>
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<tr>
<td>Chemicals</td>
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<table>
<thead>
<tr>
<th>CMCT</th>
<th>React with U&gt;Ψ&gt;G&gt;I on ssRNA</th>
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</thead>
<tbody>
<tr>
<td>DMS</td>
<td>Methyate G&gt;A&gt;C on ssRNA</td>
</tr>
<tr>
<td>Diethyl pyrocarbonate</td>
<td>React with A&gt;G</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>React with U&gt;Ψ&gt;C&gt;T</td>
</tr>
<tr>
<td>Limited hydrolysis</td>
<td>Formamidine</td>
</tr>
<tr>
<td></td>
<td>Hot water (80°C)</td>
</tr>
<tr>
<td></td>
<td>2D acrylamide gel electrophoresis</td>
</tr>
<tr>
<td></td>
<td>2D homochromatography</td>
</tr>
<tr>
<td>Fractionation</td>
<td>2D electrophoresis</td>
</tr>
<tr>
<td></td>
<td>2D paper chromatography</td>
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<tr>
<td></td>
<td>2D thin layer chromatography</td>
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</tbody>
</table>

The reaction was carried out in 0.1 M sodium acetate buffer at pH 5 with freshly prepared NaIO 4 in the dark for 1 hour and precipitated the RNA with ethanol. The RNA was redissolved in the same buffer and treated with ethylene glycol to destroy excess NaIO 4. The RNA was precipitated with ethanol and redissolved in 0.1 M sodium phosphate buffer, pH 7.7, and treated with radioactive [3H]-KBH 4 [38]. These reaction products would have tritium labeling in cis-alcohols from cis-aldehyde oxidation products of the 2' and 3' hydroxyls of ribose, assuming all 3' ends of RNA have accessible 2' and 3' OH groups (Figure 12).

The labeled 4–8S RNAs were separated by preparative polyacrylamide gel electrophoresis (Figure 13) and DEAE-Sephadex column chromatography (Figure 14) to purify individual snRNAs (U1, U2, U3, 4.5S RNA I, II, and III, 5S RNA I, II, and III).

Alkaline hydrolysis of these RNAs produced 3' end nucleoside trialcohol derivatives (Table 9) which were subsequently identified by paper chromatography.

The RNA that appeared to be pure for sequencing was 4.5S RNA I which had 87.4% U at the 3' terminus and only 6.5% unknown radioactivity at the origin. Unexpectedly, U1, U2, U3, 4.5S RNA II, and some of 5S RNA (5S RNA III/U5) had ~50% labeling in alkaline-resistant fragments that did not move as nucleoside derivatives. The 4.5S RNA III was not labeled by this procedure suggesting a blocked 3' end (Figure 14). The U1, U2, and U3 RNAs were labeled with tritium, digested with RNase A, and separated on a DEAE-Sephadex column (Figure 15).

The oligonucleotides were digested with T1 RNase and rechromatographed, and only the U3 oligonucleotide was shortened by one nucleotide, indicating the presence of one G adjacent to RNase A susceptible pyrimidine [80]. In the course of sequencing U1, U2, U3 RNAs, it was found that the oligonucleotides with m3,2,7G was coming from the 5' end segments. The only way 2'3' hydroxyls could be at 5' end was 5'3'-pyrophosphate linkage to the rest of the RNA molecules [36]. The RNase A and T1RNase resistant oligonucleotides were digested with various enzyme combinations including snake venom phosphodiesterase, alkaline phosphatase, P1 RNase, T2 RNase, and U2 RNase into nucleosides. The component nucleosides were identified by mass spectrometry, U.V. spectroscopy, HPLC (high pressure liquid chromatography), paper chromatography, and thin layer chromatography [12, 16, 37, 58].
7.2. Tritium Labeling of Nucleosides. The purified RNAs were digested with RNase A, snake venom phosphodiesterase, and alkaline phosphatase at pH 8.0, 37°C for 6 hours into nucleosides. The digest was treated with a 2X molar excess of NaIO₄ and labeled with [³H]-KBH₄ at pH 6 for 2 hours in the dark to produce trialcohol derivatives of nucleosides. All nucleosides with base modifications, except 2’-O-ribose modified, were labeled with tritium. The tritium-labeled trialcohol derivatives were separated by two-dimensional TLC (thin layer chromatography) on cellulose thin layers (Figure 5) [81]. The first dimension used a solvent of acetoniitrile, ethylacetate, n-butanol, isopropanol, 6N aqueous ammonia (7:2:1:1:2.7); the second dimension used a solvent of tert-amylic alcohol, methylethylketone, acetoniitrile, ethylacetate, water, formic acid (sp.gr. 1.2) (4:2:1.5:2:1.5:0.18) [81, 82].

Table 8: Distribution of ESE, 5’ splice sites, branch sites, and 3’ splice sites. The ESE, 5’ splice site, branch site, and 3’ splice site in 4.5S RNA I and Alu elements in FMR1 gene transcript are screened by ESE finder (version 3) [13]. For this comparison, the number of motifs is calculated per 100 nucleotides. The motif patterns in Alu elements are all very much alike and the 4.5S RNA I resembles them. A difference is found in that 5’ splice sites in (+) Alu are more than in (−) Alu and 3’ splice sits are more in (−) Alu than in (+) Alu.

<table>
<thead>
<tr>
<th></th>
<th>SF2/ASF</th>
<th>SC35</th>
<th>SRp40</th>
<th>SRp55</th>
<th>Total</th>
<th>5’SS</th>
<th>BS</th>
<th>3’SS</th>
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<tr>
<td>Novikoff 4.5S RNA I (96 nt)</td>
<td>3.65</td>
<td>3.13</td>
<td>6.25</td>
<td>1.04</td>
<td>14.07</td>
<td>0</td>
<td>10.4</td>
<td>2.08</td>
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<td>Human FMR1 Alu1(+) (252 nt)</td>
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<td>7.54</td>
<td>6.35</td>
<td>1.59</td>
<td>20.44</td>
<td>3.97</td>
<td>9.13</td>
<td>5.56</td>
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<td>4.41</td>
<td>1.69</td>
<td>2.03</td>
<td>12.54</td>
<td>2.37</td>
<td>10.9</td>
<td>2.37</td>
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<td>Human FMR1 Alu5(+) (246 nt)</td>
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<td>5.28</td>
<td>4.47</td>
<td>1.22</td>
<td>16.66</td>
<td>4.47</td>
<td>8.54</td>
<td>2.44</td>
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<tr>
<td>Human FMR1 Alu7(+) (290 nt)</td>
<td>6.21</td>
<td>2.41</td>
<td>3.10</td>
<td>0.34</td>
<td>12.06</td>
<td>3.79</td>
<td>10.3</td>
<td>2.07</td>
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<tr>
<td>Human FMR1 Alu8(+) (288 nt)</td>
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<td>3.47</td>
<td>3.82</td>
<td>0.69</td>
<td>13.19</td>
<td>3.47</td>
<td>8.68</td>
<td>2.43</td>
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<tr>
<td>(+) Alu Av.</td>
<td>5.30</td>
<td>4.62</td>
<td>3.89</td>
<td>1.17</td>
<td>14.98</td>
<td>3.61</td>
<td>9.51</td>
<td>2.97</td>
</tr>
<tr>
<td>Human FMR1 Alu2(−) (298 nt)</td>
<td>3.69</td>
<td>6.04</td>
<td>5.03</td>
<td>1.34</td>
<td>16.10</td>
<td>0.67</td>
<td>14.1</td>
<td>4.70</td>
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<tr>
<td>Human FMR1 Alu3(−) (285 nt)</td>
<td>4.92</td>
<td>7.02</td>
<td>5.61</td>
<td>2.11</td>
<td>19.66</td>
<td>3.16</td>
<td>13.7</td>
<td>4.56</td>
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<td>Human FMR1 Alu6(−) (290 nt)</td>
<td>4.31</td>
<td>5.52</td>
<td>4.48</td>
<td>1.38</td>
<td>15.69</td>
<td>2.76</td>
<td>13.5</td>
<td>4.48</td>
</tr>
<tr>
<td>(−) Alu Av.</td>
<td>4.31</td>
<td>6.19</td>
<td>5.04</td>
<td>1.61</td>
<td>17.15</td>
<td>2.20</td>
<td>13.8</td>
<td>4.58</td>
</tr>
<tr>
<td>Human FMR1 Total Alu (+&amp;−) Av.</td>
<td>4.93</td>
<td>5.21</td>
<td>4.32</td>
<td>1.34</td>
<td><strong>15.80</strong></td>
<td>3.08</td>
<td>11.1</td>
<td>3.58</td>
</tr>
</tbody>
</table>

Figure 9: The mass spectra of trimethylguanosine [12]. The synthetic m32,2,7G and unknown nucleoside from U2 RNA were trimethylsilylated and subjected to LKB 9000 gas chromatograph-mass spectrometer. The mass spectrum of the unknown nucleoside from U2 RNA was identical to synthetic m32,2,7G.
Presence of ESE, branch sites and splice sites in 4.5S RNA I (Novikoff)

**Table 9:** The snRNA 2′ and 3′-OH labeling by NaIO₄ oxidation and [³H]-KBH₄ reduction [38]. The total 4–7S RNA from rat Novikoff hepatoma cell nuclei was labeled with [³H] by oxidation with NaIO₄ followed by [³H]-KBH₄ reduction (Figure 12). Individual RNA species were purified by gel electrophoresis (Figure 13). The RNA samples were hydrolyzed with 0.3 N KOH, and hydrolysates were chromatographed on whatman 3MM paper according to de Wachter and Fiers [55]. The radioactivities at the origin (22% for 5S RNA, 54.1% for U1 RNA, 49.7% for U2 RNA, and 50.6% for U3 RNA) represent % of total radioactivity applied and they represent the 5′ end labeling which was later elucidated by many enzymatic methods described in the text. The radioactivities moved by chromatography with standard nucleoside derivatives are the % of total in nucleosides derivatives. The A′ U′ G′ C′ represent trialkohol derivatives of nucleosides.

<table>
<thead>
<tr>
<th>RNA Species</th>
<th>Radioactivity at origin (5′)</th>
<th>A′</th>
<th>U′</th>
<th>G′</th>
<th>C′</th>
</tr>
</thead>
<tbody>
<tr>
<td>4S RNA</td>
<td>10.9</td>
<td>89.0</td>
<td>3.2</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>4.5S RNA</td>
<td>15.8</td>
<td>11.2</td>
<td>79.7</td>
<td>4.0</td>
<td>5.1</td>
</tr>
<tr>
<td>4.5S RNA I</td>
<td>6.5</td>
<td>6.1</td>
<td>87.4</td>
<td>4.7</td>
<td>1.8</td>
</tr>
<tr>
<td>4.5S RNA II</td>
<td>30.9</td>
<td>13.1</td>
<td>80.2</td>
<td>4.7</td>
<td>2.4</td>
</tr>
<tr>
<td>5S RNA</td>
<td>22.0</td>
<td>11.4</td>
<td>75.5</td>
<td>6.0</td>
<td>7.0</td>
</tr>
<tr>
<td>U1 RNA</td>
<td>54.1</td>
<td>6.0</td>
<td>13.4</td>
<td>77.7</td>
<td>3.0</td>
</tr>
<tr>
<td>U2 RNA</td>
<td>49.7</td>
<td>61.5</td>
<td>6.0</td>
<td>4.3</td>
<td>28.2</td>
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<tr>
<td>U3 RNA</td>
<td>50.6</td>
<td>53.8</td>
<td>22.6</td>
<td>9.8</td>
<td>13.7</td>
</tr>
</tbody>
</table>

7.3. [³²P] Labeling of RNA. The Novikoff hepatoma cells were transplanted intraperitoneally into male albino rats of the Holtzman strain weighing 200–250 g, obtained from Cheek Jones Company (Houston, Tex). After 5–6 days, the cells were harvested and washed with NKM solution (0.13 M NaCl, 0.005 M KCl, and 0.008 M MgCl₂). Twenty milliliter (packed volume) of cells was incubated with 500 mCi of [³²P]-orthophosphate in 1 liter of medium (phosphate free modified Eagle’s minimal essential medium) for 9–16 hours [83]. Nuclear RNA was purified by sucrose gradient centrifugation, gel electrophoresis, and column chromatography [38]. The purified RNA was hydrolyzed with 0.3 N KOH, and alkaline-resistant oligonucleotides were separated on DEAE-Sephadex. The alkaline resistant dinucleotides were collected, treated with alkaline phosphatase, and identified by two-dimensional chromatography (Figure 16).

The summary of modified nucleotides is in Table 1 [84].

8. Structural Determination of 5′ Oligonucleotides

The structures of the 5′ ends of U1 RNA, U2 RNA, U3 RNA, and 5S RNA III (U5) are determined by the characteristics of chemical reactions and enzymatic susceptibilities (Figure 17).

8.1. U1 RNA 5′ End Oligonucleotide. The U1 RNA labeled with [³H] by NaIO₄ and [³H]-KBH₄, digested with RNase
(1) Human HeLa U1A RNA (2) Rat Novikoff U1 RNA (Involved pre-mRNA splicing)

1. m$_{2,3,7}$GpppAmUmAC'T*ACCU
2. m$_{2,3,7}$GpppAmUmAC'T*ACCU

(3) CAGCAAGGUG GUUUCGCCAG GCGAGGCUU AUCCAUUGCAm CUCGGGAU
(4) AGAGGAACUUG GUUUCGCCAG GCGAGGCUU AUCCAUUGCAm CUCGGGAU

(1) GCUGAACCUC GCGAUAUCCCC AAAUGUGGG AAACUCGACU GCAUAUUUG
(2) GUGCAGCUG AGCCCUGGCU UUUCUCUCUA AUGCAGGAGA AACUGGGG

(1) UGGUAGUGGG GAGUGCUGUU CGCGCUUCC CCUG–OH (164) [15]
(2) UAUUUUGUGG UAGUCGCGGG GACUGUUGUC GCUCUCUCG–OH (170) [16]

Pre-mRNA 5' splice site binding region  Sm protein binding region

(3) Human HeLa U2 RNA (4) Rat Novikoff U2 RNA (Involved in pre-mRNA splicing)

(3) m$_{2,3,7}$GpppAmUmCGCUUCG GmGmCUUUUGmG CUAAGmACUAAm
(4) m$_{2,3,7}$GpppAmUmCGCUUCG GmGmCUUUUGmG CUAAGmACUAAm

(3) CUCCAUUCCGA GGACAUUAUA UUAAGUGAU UUUAGGAGCA GGGAGAGGA
(4) CUCCAUUCCGA GGACAUUAUA UUAAGUGAU UUUAGGAGCA GGGAGAGGA

Branch point binding region  Sm protein binding region

(5) Human Placenta U3 RNA (6) Rat Novikoff U3B RNA (Involved in rRNA production)

(5) m$_{2,3,7}$GpppAmAmGACUAUAC UUUACGCUUUUAU AUUGGUACU
(6) m$_{2,3,7}$GpppAmAmGACUAUAC UUUACGCUUUUAU AUUGGUACU

(5) AGAGGAAGUUU CUCUGAACGU GUAGAGCACC GAAAACCAG AGGAAGAGAG
(6) AGAGGAAGUUU CUCUGAACGU GUAGAGCACC GAAAACCAG AGGAAGAGAG

(5) UGAGCGUUUU CUCCUGACCCG CGUUCUGCCG UUGCUUGCCG
(6) UGAGCGUUUU CUCCUGACCCG CGUUCUGCCG UUGCUUGCCG

(5) CAACUUGCUU GAGUACGCUUC UCUCUCCGA UUGGGGAGUG
(6) AGCUUGCUUC UCUCUCCGA UUGGGGAGUG

(5) AGAGGGAGAG AACCGCUUUCA GAGUGGU–OH (217) [19,20]
(6) AGAGGGAGGAA CAACUUGCUU GUGGA–OH (215) [21]

(7) Human HeLa U4A RNA (8) Rat Novikoff U4 RNA (Involved in pre-mRNA splicing)

(7) m$_{2,3,7}$GpppAmGmC*UUGmGC AGUGCAGUA UCGUAGCCAA UAGGGUUAA
(8) m$_{2,3,7}$GpppAmAC*UUGmGC AGUGCAGUA UCGUAGCCAA UAGGGUUAA

(7) CCCGAGCCGCC AUUAUGUUGCU AYUGmAAACCU U*YCCCAAA* YCCGCCGGUG
(8) GAGGCCGCU UAUUGGAAU UGmAAACCU UYCCCAAA* YCCGCCGGUG

(7) ACUGC–OH (144–145) [22,23]

(a) Figure 11: Continued.
A, showed enzyme-resistant oligonucleotide eluting close to the pentanucleotide region on a DEAE column (Figure 15). The 5’ oligonucleotide was analyzed by UV, [3H], and [32P] methods.

8.1.1. The UV Analysis. The 5’ oligonucleotides from U1 RNA, obtained by RNase A and RNase T1, were digested with snake venom phosphodiesterase and alkaline phosphatase. The nucleosides produced were separated on HPLC (high pressure liquid chromatography) [strongly basic cation exchange (quaternary amine)]. As shown in Figure 18, the amount of nucleoside ratio was 1.0, 1.2, 1.2, 0.7, and 0.9 for Am, A, Um, m32,2,7G, and C, respectively, for U1 5’ oligonucleotide.
8.1.2. The \(^{3}H\) Method. The \(^{3}H\)-labeled U1 RNA 5′ oligonucleotide, following digestion with snake venom phosphodiesterase and alkaline phosphatase, was separated by chromatographic methods with standards. Two-dimensional TLC (thin layer chromatography) and paper chromatography demonstrated that the \(^{3}H\) labeled compound is a trimethylguanosine derivative (Figure 19).

8.1.3. \(^{32}P\)-Labeled 5′ Oligonucleotide from U1 RNA. The \(^{32}P\)-labeled RNA was digested with T₂ and U₂ RNase, and digestion products were separated by two-dimensional electrophoresis. The first dimension was on cellogel at pH 3.5, and the second dimension was on DEAE paper at pH 3.5 (Figure 20).

Spot “a” was eluted and treated with alkaline phosphatase and chromatographed with GMP, GDP, and GTP standards. The \(^{32}P\)-labeled 5′ oligonucleotide was chromatographed in the GTP region on a DEAE-Sephadex column (Figure 21).

The oligonucleotide peak from the GTP region was digested with snake venom phosphodiesterase and separated by electrophoresis in the first dimension followed by chromatography on second dimension (Figure 22).

The \(^{32}P\) activity ratio was 1.00, 1.11, 1.25, 0.53, and 1.14 for pm3,2,7G, pAm, pUm, pA, and Pi, respectively. The peak from the GTP region in Figure 21 digested with RNase P1 produced pUm, pA (peak a in Figure 23), and cap core m3,2,7GpppAm (peak b in Figure 23). Table 10 shows the radioactivity distribution in peaks a and b in Figure 23.

For the analysis of a number of phosphates in cap core (peak b), the cap core was treated with NaIO₄ and aniline to remove m3,2,7Gbγβ-elimination reaction (Figure 24).

The product was chromatographed on a DEAE column with standard AMP, ADP, and ATP. The product was eluted close to ATP, indicating that it is pppAm. This experiment proved that the 5′ oligonucleotide structure is m₃,2,7GpppAmpUmpApCp.
8.2. U2 RNA 5' End Oligonucleotide. The U2 RNA labeled with NaIO$_4$ and [$^3$H]-KBH$_3$ was digested with RNase A. The labeled oligonucleotide eluted around the tetranucleotide region (Figure 15). The 5' oligonucleotide was analyzed by UV, [$^3$H], and [$^{32}$P] methods.

8.2.1. UV Analysis. The 5' oligonucleotide obtained by complete RNase A digestion was analyzed for its base composition. The purified 5' oligonucleotide was digested with snake venom phosphodiesterase followed by alkaline phosphatase. The digestion product (nucleosides) was separated by HPLC. The composition was Am, Um, C, and m$_3^{2,2,7}$G in a ratio of 1.0, 1.3, 1.1, and 0.96, respectively, (Figure 18) [12]. These nucleosides were also separated by two-dimensional TLC in a borate system. Um and Am migrated through the butanol-boric acid while the m$_3^{2,2,7}$G and C, which form complexes with borate, were retarded in the butanol-boric acid phase (Figure 25).
The UV spectra of pm^3,2,7G were typical of a trimethyl G nucleotide (Figure 8). The mass spectrometry of the unknown nucleoside from U2 RNA 5’ fragment was identified as m^3,2,7 trimethylguanosine (Figure 9).

8.2.2. ^3H-Labeled U2 RNA 5’ Oligonucleotide. The purified U2 RNA, labeled with NaIO4 and [3H]-KBH4 methods, was digested with RNase A and 5’ oligonucleotide purified by DEAE-Sephadex column chromatography (Figure 15). The purified 5’ oligonucleotide was digested with snake venom phosphodiesterase followed by alkaline phosphatase. The nucleosides obtained were separated on two-dimensional TLC [12] and 3MM paper chromatography. The tritium-labeled compound was identified as a trialcohol derivative of m^3,2,7G (Figure 26).

8.2.3. ^32P-Labeled U2 RNA 5’ Oligonucleotide. The ^32P-labeled U2 RNA was digested with T1 RNase or RNase A. Half of each 5’ oligonucleotide was digested with alkaline phosphatase. Oligonucleotides were subsequently digested with snake venom phosphodiesterase, and the resulting 5’ nucleotides were separated first by electrophoresis and second by chromatography (Figure 27). The ratio of ^32P counts is shown in Table 11.

From these data the 5’ end oligonucleotide from U2 RNA has been deduced to be m^3,2,7GpppAmpUmpCpGp.

8.3. U3 RNA 5’ End Oligonucleotide. The ^3H-labeled U3 RNA was digested with RNase A and or T1 RNase. The ^3H-labeled 5’ oligonucleotide obtained by RNase A digestion was eluted in the hexanucleotide region (Figure 15). The ^32P-labeled U3 RNA digested with T2 and U2 RNA produced 2 spots that were separated by two-dimensional electrophoresis (Figure 29).

8.3.1. UV Analysis. The 5’ oligonucleotide obtained from U3 RNA by digestion with RNase A and T1 RNase was isolated by column chromatography. The purified 5’ oligonucleotide was digested with snake venom phosphodiesterase and alkaline phosphatase. The nucleosides obtained were subjected to HPLC. The molar ratios of m^3,2,7G, Am, A, and G were 1.0, 1.7, 1.1, and 1.0, respectively (Figure 18).

8.3.2. ^3H Analysis. The intact U3 RNA, labeled with NaIO4 and [3H]-KBH4 methods, was digested with RNase A and
Trimethyl guanosine caps of Novikoff hepatoma cell snRNAs cleavage sites by various enzymes

<table>
<thead>
<tr>
<th>CAP structure of U1 RNA</th>
<th>m3,2,7-GpppAm pUm pApCp</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP structure of U2 RNA</td>
<td>m3,2,7-GpppAm pUm pCp</td>
</tr>
<tr>
<td>CAP structure of U3 RNA</td>
<td>m3,2,7-GpppAm pUm pApCp</td>
</tr>
<tr>
<td>5S RNA III (U5 RNA)</td>
<td>m3,2,7-GpppAm pUm pApCp</td>
</tr>
</tbody>
</table>

Nuclease
- T1 RNase
- PI nuclease
- Snake venom phosphodiesterase
- T2 RNase
- U2 RNase
- RNase A
- Bacterial alkaline phosphatase

**Figure 17:** Characterization of m3,2,7-G caps of snRNAs U1, U2, U3, and U5. The enzyme susceptible bonds are indicated with arrows. The split arrows indicate that some bonds without 2'′-O-methylation can be cleaved but the ones with 2′-O-methylated ribose are not.

**Table 11:** The analysis of [32P]-labeled U2 RNA 5′ oligonucleotide [12]. The 5′ ends obtained from uniformly [32P]-labeled U2 RNA were digested with T1 RNase or RNase A and isolated by two-dimensional electrophoresis (cellulose acetate at pH 3.5 followed by DEAE paper electrophoresis). The 5′ oligonucleotides were digested with snake venom phosphodiesterase before and after removal of 3′ phosphate with bacterial alkaline phosphatase. The products were separated as in Figure 27. The radioactivity ratios are listed.

<table>
<thead>
<tr>
<th>5′ fragment from U2 RNA</th>
<th>Pi</th>
<th>pUm</th>
<th>pAm</th>
<th>pC</th>
<th>pG</th>
<th>pGp</th>
<th>32P ratio in nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′ oligo from T1 digestion</td>
<td>1.58</td>
<td>1.33</td>
<td>0.91</td>
<td>0.90</td>
<td>1</td>
<td>1.48</td>
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<tr>
<td>Alkaline phosphatase digested 5′ oligo</td>
<td>1.22</td>
<td>1.43</td>
<td>0.98</td>
<td>0.90</td>
<td>1</td>
<td>0.93</td>
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<td>5′ oligo from RNase A digestion</td>
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<td>1.25</td>
<td>1</td>
<td>1.08</td>
<td>1.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase digested A 5′ oligo</td>
<td>0.93</td>
<td>1.45</td>
<td>0.94</td>
<td>0.77</td>
<td>1</td>
<td></td>
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</table>

From these data, obtained by UV, [3H], and [32P] experiments, the U3 RNA 5′ oligonucleotide sequence has been deduced to be m3,2,7-GpppAmpA(m)pApGpCp.

8.3.3. [32P] Analysis. The [32P]-labeled U3 RNA digested by T1 RNase and U2 RNase was separated by two-dimensional electrophoresis (Figure 29). The enzyme-resistant oligonucleotides 11A and 11B were eluted from the paper and treated with alkaline phosphatase. The products were chromatographed on DEAE-Sephadex A-25 with GMP, GDP, and GTP markers. The 11A (cap II) was eluted at GTP region and 11B (cap I) was eluted at GDP region, indicating that 11B has one nucleotide less than 11A (Figure 31).

From these data, obtained by UV, [3H], and [32P] experiments, the U3 RNA 5′ oligonucleotide sequence has been deduced to be m3,2,7-GpppAmpA(m)pApGpCp.

8.4. 5S RNA III (U5 RNA) 5′ End Oligonucleotide. The oligonucleotide sequence was deduced as in the case of U1 RNA. The structure is identical to the U1 5′ oligonucleotide m3,2,7-GpppAmpUmpApCp.
Table 12: Tgs1 interacting proteins [70–72]. The Tgs1 (trimethylguanosine synthase 1) interacting proteins are listed. It is interacting with proteins involved in many aspects of RNA metabolism such as transcription, spliceosome assembly, maturation, and modification.

<table>
<thead>
<tr>
<th>Tgs1 interacting proteins [70]</th>
<th>Spliceosome assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription apparatus (regulators of RNA polymerase II transcription)</td>
<td>(1) Mud1 (yeast homolog of U1A)</td>
</tr>
<tr>
<td>(1) Rpn4 (TF; Proteasome subunits and U2 snRNA gene)</td>
<td>(2) Mud2 (yeast homolog of U2AF65)</td>
</tr>
<tr>
<td>(2) Spt3 (SAGA complex)</td>
<td>(3) Nam8 (Mud15) (homolog of TIA-1)</td>
</tr>
<tr>
<td>(3) Srb2 (mediator complex)</td>
<td>(4) Brr1 (snRNPs)</td>
</tr>
<tr>
<td>(4) Soh1 (Med31; mediator complex)</td>
<td>(5) Lea1 (U2A; U2snRNPs)</td>
</tr>
<tr>
<td>(5) Swr1 (ATPase; binds to 5' end of yeast transcription units)</td>
<td>(6) Ist3 (Snu17; U2 snRNPs)</td>
</tr>
<tr>
<td>(6) Htz1 (H2AZ) (binds to 5' end of yeast transcription units)</td>
<td>(7) Isy1 (Ntc30; interacts with Prp19)</td>
</tr>
<tr>
<td>(7) CBP (binds to human PIMT/Tgs1)</td>
<td>(8) Cwc21 (component of Celf1 complex)</td>
</tr>
<tr>
<td>(8) P300 (binds to PIMT/Tgs1)</td>
<td>(9) Bud13 (Cwc26) (Celf1 complex)</td>
</tr>
<tr>
<td>(9) PRIP (binds to PIMT/Tgs1; [71])</td>
<td>(10) SMN (HeLa cell) [72]</td>
</tr>
<tr>
<td>RNA end processing and decay</td>
<td>RNA modifying factors</td>
</tr>
<tr>
<td>(1) Trf4 (Pap2; catalytic subunit of TRAMP)</td>
<td>(1) SmB (snRNPs; interacts with</td>
</tr>
<tr>
<td>(2) Lsm1 (decapping complex)</td>
<td>YNL187/Swt21</td>
</tr>
<tr>
<td>(3) Pat1 (decapping complex)</td>
<td>(2) SmDL (snRNPs)</td>
</tr>
<tr>
<td>RNA modifying factors</td>
<td>(3) Cbf5 (snoRNPS)</td>
</tr>
<tr>
<td>(1) SmB (snRNPs; interacts with YNL187/Swt21)</td>
<td>(4) Nop58 (snoRNPs)</td>
</tr>
<tr>
<td>(2) SmDL (snRNPs)</td>
<td>(5) Mrm (RNA 2'-'O-methyltransferase)</td>
</tr>
</tbody>
</table>

9. RNA Signature Modifications for Different RNA Classes

9.1. End Modifications

9.1.1. 5' End

(a) According to Chemical Nature of Caps

5' Trimethylguanosine cap for the snRNA,
5' 7-methylguanosine cap for the mRNA,
5' 2,7 dimethylguanosine cap of virus and nematode RNAs
5' mpppG of U6 RNA.

(b) According to Flanking Nucleotide Modification of Caps. (See Table 13).

(c) 5' End Uncapped RNA. (pppNp) for primary transcripts such as 4.5S RNA I, 5S RNA, and Alu RNA. (pNp) 5' end for processed RNAs such as Alu RNA, 5S RNA, tRNA, YRNA.

9.1.2. 3' End

3' 2'-'O-methylated; 4.5S RNA III
3' poly-A; mRNA, IncRNA
3' poly-U; polymerase III transcripts such as 4.5S RNA I, 5S RNA, and others
3' CCA; tRNA, U2 RNA.

9.2. Internal Modifications. The most colorful modifications are in tRNAs that contain methyl, formyl, acetyl, isopentyl, threonyl, carbamoyl, and other groups and modifications by pseudouridylation, deamination, reduction, or thiolation. Focusing on recent findings for snRNA, m32,2,7G capping reactions are very interesting because trimethylguanosine is found only in noncoding RNA cap structures, although some nematode mRNA species also contain m32,2,7G caps. The snRNAs are less abundant (10^5 copies) than ribosomal RNA or tRNA (10^6 copies). Isolating large amount of RNA can be a hurdle to overcome. Massive preparative procedures and syntheses were pivotal for the thorough analysis of these modifications. The 2'-'O-modifications occur mostly internally, and 3' Um was also found in 4.5S RNA III. The RNA ribose with 2'-'O-methylation confers resistance to enzymatic digestion by such enzymes as RNase A, RNase T1, RNase U2, and RNase T2. They are also resistant to alkaline hydrolysis, and the alkaline hydrolysates can be separated into di-, tri-, and tetranucleotides by column chromatography and then by two-dimensional paper chromatography (Figure 16). Other enzymes which can cleave 2'-'O-methylated nucleotides are snake venom phosphodiesterase, P1 nuclease, and spleen phosphodiesterase. These are valuable tools for sequencing.

10. Presence of m3,2,7G Caps in RNA Species

10.1. Nucleolar RNA. Initially, the m3,2,7G cap containing snoRNA was found in U3 RNA [36]. Since then C/D snoRNA and H/ACA snoRNA have been discovered exponentially. The snoRNAs are transcribed from monocistronic as well
as polycistronic independent positions as well as intronic regions of mRNA, especially the genes coding ribosomal proteins. In vertebrates, there have been >76 snoRNAs that have been reported, but only U3, U8, and U13 snoRNAs have been reported to have m$_{3,2,7}$G caps [33, 88]. In yeast, there are at least 17 m$_{3,2,7}$G cap containing snoRNAs out of more than 76 snoRNAs. It was also reported that some snoRNA precursors, such as pre-snoRNAs 50, 64, and 69, have the m$_{3,2,7}$G cap, but mature snoRNA 50, 64, and 69 do not have m$_{3,2,7}$G caps. The maturation process cleaves the 5' fragment by Rnt1 (RNase III like enzyme), and trimming is performed by 5' → 3' exonuclease Xrn1 and Rat 1 [89].

10.2. Spliceosomal snRNAs. These include U1, U2, U4, U5, and U6 snRNAs. All of these except U6 contain the m$_{3,2,7}$G cap, and U6 has the mpppG cap instead. They are present in complexes as RNP with proteins specific for each RNA as well as some common snRNP proteins such as the Sm proteins. Functionally, U1 RNP acts at 5' splice sites and U2 RNA at branch sites including 3' splice sites. U4, U5, and U6 snRNAs enter the spliceosomal intermediate as a tri-snRNP complex.

10.3. Human Telomerase RNA (hTR). Human telomerase RNA has a structure containing the H/ACA motif with 8 conserved regions (CR 1–8) [92]. The CR7 contains the CAB...
### Table 13: Cap variations of flanking nucleotide modifications.

<table>
<thead>
<tr>
<th>Type</th>
<th>Trimethylguanosine cap</th>
<th>7-Methylguanosine cap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 0</td>
<td>( m_3^{2,2,7}GpppN^- )</td>
<td>( m_7^{7}GpppN^- )</td>
</tr>
<tr>
<td>Type I</td>
<td>( m_3^{2,2,7}GpppNmN^- )</td>
<td>( m_7^{7}GpppNmN^- )</td>
</tr>
<tr>
<td>Type II</td>
<td>( m_3^{2,2,7}GpppNmNmN^- )</td>
<td>( m_7^{7}GpppNmNmN^- )</td>
</tr>
<tr>
<td>Type III</td>
<td>( m_3^{2,2,7}GpppNmNmNmN^- )</td>
<td>( m_7^{7}GpppNmNmNmN^- )</td>
</tr>
<tr>
<td>Type IV</td>
<td>( m_3^{2,2,7}GpppNmNmNmNmN^- )</td>
<td>( m_7^{7}GpppNmNmNmNmN^- )</td>
</tr>
</tbody>
</table>

---

**Figure 19**: Identification of \( m_3^{2,2,7}G' \) [16]. (a) Fluorogram of two-dimensional thin layer chromatography of \([^3]H\)-labeled nucleoside trialcohol derivative (N') released from U1 RNA 5' fragment. It was identified as \( m_3^{2,2,7}G' \) with standard. (b) Fluorogram of chromatographic separation of \([^3]H\)-labeled nucleoside trialcohol derivative on Whatman 3MM paper. The \([^3]H\)-labeled compound was identified as \( m_3^{2,2,7}G' \) with standard.

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**Figure 20**: 2D map of U1 RNA digested with T2 RNase and U2 RNase [16]. The U1 RNA uniformly labeled with \([^{32}P]\) was digested with T2 RNase and U2 RNase. The resistant 5' fragment (spot "a") was separated from the rest of the hydrolysate by two-dimensional electrophoresis. The first dimension was on cellogel at pH 3.5, and the second dimension was on DEAE paper in 5% acetic acid-NH\(_4\) acetate at pH 3.5.

---

10.4. *C. elegans* SL RNA. *C. elegans* has mRNA with the m7G cap as well as \( m_3^{2,2,7}G \) cap, and the expression is regulated differentially. The genes for protein coding are monocistronic as well as polycistronic, and introns are much smaller than observed in mammalian cells. The polycistronic genes contain 2–8 operonic genes regulated by the same promoters. Some gene products are not processed, and others are spliced by cis-splicing as well as transsplicing. The transsplicings are carried out by SL RNA 1 or SL RNA 2. The approximately 110 SL RNA 1 genes are in tandem in chromosome V. The SL RNA 2 is derived from SL RNA 1 and there are \( \sim18 \) dispersed genes with a variety of variant SL2 RNAs (some are called SL3, SL4, etc.). They are all 100–110 nucleotide long and contain \( m_3^{2,2,7}G \) caps and Sm protein binding sites. These pre-mRNAs, containing 5' outron (monocistronic and 5' first gene in polycistronic operonic genes), are transspliced by SL RNA 1 and internal operonic pre-mRNAs are mostly transspliced by SL RNA 2 and these genes have typically U-rich sequence containing \( \sim100\) bp spacers between two cleavage sites. The internal
Figure 21: DEAE column chromatography of U1 5′ oligonucleotide [16]. Spot “a” in Figure 20 was eluted and digested with alkaline phosphatase. The product was chromatographed on DEAE-Sephadex A-25 with GMP, GDP, and GTP. The fragment chromatographed at GTP region.

mRNA gene of polycistronic operonic genes, lacking a spacer, is transspliced always by SL RNA I [95, 96]. The transspliced mRNA contains a m₃G₂,₇G cap containing 22 nucleotides of SL RNA at their 5′ ends. The SL RNA (splice leader RNA) has a m₃G₂,₇G cap and Sm protein binding sites. The nematode *C. elegans* has 5 eIF4E isoforms of cap binding proteins. They are IFE-1 (m₇G cap and m₃G₂,₇G cap binding), IFE-2 (m₇G cap binding, but competed by the m₃G₂,₇G cap), IFE-3 (m₇G cap binding only), IFE-4 (m₇G cap binding only), and IFE-5 (m₇G cap and m₃G₂,₇G cap binding). The homolog amino acids W56 and W102 stacking the m₇G cap in IFE-1 are W51 and W97 in IFE-3 and W28 and W74 in IFE-5 (Figure 32).

The differences in 3-4 loop configuration between IFE-5 and IFE-3 are N64Y/V65L. The changes in IFE-5 amino acid asparagine 64 to tyrosine and valine 65 to leucine change binding properties more to m₇G cap binding than to m₃G₂,₇G cap binding. IFE-5 has 4 cysteines, and its conformation is governed by disulfide bond formation. It is suggested that the cap binding cavity is altered to produce a smaller cavity that discriminates against the m₃G₂,₇G cap binding [85].

11. Synthesis of m₃G₂,₇G Cap

modifications. Trimethyl G caps are present in snRNAs involved in splicing and also in snoRNA involved in rRNA processing and modifications such as Ψ formation (H/ACA snoRNA) or 2′-O-methylation (C/D snoRNA). These include U1, U2, U4, and U5 spliceosomal RNAs, and U3, U8, and U13 nucleolar RNAs. Recently, telomerase RNA (*S. cerevisiae* TLC1) has also been reported to have
Figure 24: The β-elimination of cap core [16]. The cap core (peak b from Figure 23) was treated with NaIO₄ and aniline to remove m₃G by β-elimination reaction. The remaining nucleotide was chromatographed in the ATP region indicating it is pppAm. This proved that the cap core was m₃GpppAm.
Um and Am migrated through the butanol-boric acid while the $m_7^2,2,7G$ and C, which form borate complexes, were retained in the butanol-boric acid phase.

Figure 25: Two-dimensional chromatography in borate system [12]. The nucleoside mixture from U2 RNA 5′ oligonucleotide (RNase A product) was obtained by digestion with snake venom phosphodiesterase and alkaline phosphatase. The resulting nucleosides were separated with the borate system. Um and Am migrated through the butanol-boric acid while the $m_7^2,2,7G$ and C, which form borate complexes, were retained in the butanol-boric acid phase.

11.1. The $m^7G$ Cap Formation. The RNA polymerase initiates the RNA transcription with 5′ triphosphate nucleotides and in a majority with purine nucleotides of ATP or GTP. The capping reaction in a polymerase II system occurs cotranscriptionally within the nascent transcript of ~30–50 nucleotides. The guanylyltransferase is attached to heptad (YSPTSPS) repeats of CTD of RNA polymerase II. It was reported with cloned mouse guanylyltransferase and synthetic heptad repeats that the serine 5 phosphorylated 6 heptad repeats stimulated guanylyltransferase activity 4-fold. Serine 2 phosphorylation also binds the guanylyltransferase but did not stimulate enzyme activity [97].

The capping enzymes contain RNA triphosphatase and RNA guanylyltransferase in the same molecule, but methylating enzymes are in different protein and occurs in separate steps.

The enzymes involved are RNA triphosphatase and RNA guanylyltransferase, which can be found in the same enzyme, catalyze removal of one phosphate from pppNp initiation nucleotide, and transfer GMP from GTP through intermediary GMP-lysine phosphamide enzyme complex. The RNA guanyl 7 methyltransferase methylates the guanine at N7 position. The RNA 2′-O-methyltransferase methylates penultimate nucleotide 2′ OH, producing the cap 1 structure. In rat liver, it has been reported that 2′-O-methylation may precede the guanosine N7 methylation [98].

The capping reactions by mammalian and shrimp capping complexes (HeLa cell, rat liver, calf thymus, and shrimp) [98] have been reported as below:

**RNA Triphosphatase and Guanylyltransferase.** The monomer of the 69–73 kDa protein has functions of RNA triphosphatase and RNA guanylyltransferase activity.

\[
\begin{align*}
\text{pppNpNpNpNp-} & \xrightarrow{\text{RNA triphosphatase}} \text{ppNpNpNpNp-} \quad (1) \\
\text{GTP + RNA guanylyltransferase} & \xrightarrow{Mg^{2+}} \text{GMP-(phosphamide)-E + ppi} \quad (2) \\
\text{GMP-E + ppNpNpNpNp-} & \xrightarrow{Mg^{2+}} \text{GpppNpNpNpNp- + RNA guanylyltransferase} \quad (3) \\
\text{GpppNpNpNpNp- + AdoMet} & \xrightarrow{\text{RNA 2′-O-methyltransferase}} \text{GpppNmpNpNpNp- + AdoHcy} \quad (4) \\
\text{GpppNmpNpNpNp- + AdoMet} & \xrightarrow{\text{RNA guanyl 7-methyltransferase}} m^7 \text{GpppNmpNpNpNp + AdoHcy} \quad (5)
\end{align*}
\]
Some of the capping enzymes (vesicular stomatitis virus, spring viremia of carp virus) use the substrate monophosphorylated 5′ end (pNpNpNpNp-) [99, 100], and 7-methylation occurs after the 2′-O-methylation has taken place.

Trimethylguanosine cap synthesis is carried out by multiple steps involving From HeLa cells, two enzymes forming cap I from cap 0 and cap II from cap I have been purified and characterized [101].

11.2. Cap I Methyltransferase. This enzyme is present in both the nucleus (29.3 units/mg) and cytoplasm (3.74 units/mg) and cap II methyltransferase is exclusively in the cytoplasm (4.62 units/mg). Cap I methyltransferase uses GpppA(pA)n, m7GpppA(pA)n, m7GpppAPgp, m7GpppAPgpUp, and RNA with type 0 cap as substrates but not m7GpppA or GpppA. The substrate required for cap I formation should be at least a trinucleotide.

The order of 7-methylation of ultimate G nucleotide and 2′-O-methylation of penultimate nucleotide is uncertain, and both pathways may occur.

11.3. Cap II Methyltransferase. This enzyme is present only in the cytoplasm and converts cap I to cap II. The mature mRNA with 5′ m7G cap and 3′ polyadenylation is then transported into the cytoplasm as a complex with CBC20/80, PHAX, and Crm1-RanGTP. The m7G cap binds to CBC20 (156 amino acids) in complex with CBC80 (790 amino acids). The crystal structure of the CBC20/80 complex in association with m7G cap has been reported [86, 87]. The CBC20 is in an unfolded form in the absence of CBC80. The CBC80 has 3 domains, each containing consecutive 5-6 helical hairpins resembling the MIF4G (middle domain of eIF4G). The CBC20 has a typical RRM motif and binds between domains 2 and 3 of CBC80. The m7G cap is sandwiched between Tyr 43 and Tyr 20. And Phe 83, Phe 85, and Asp 116 have essential role for m7G cap binding. Asp 116 and Trp 115 interact with the N2 amino group and confer specificity of the m7Gcap for other structures (Figure 33).

In the cytoplasm, the m7G cap plays a role in the initiation of translation by binding to eIF4E which complexes with eIF4A and eIF4G. The exact mechanism of exchange is not known but CBC80 has binding capacity for PHAX or eIF4G and dissociation of CBC80 from CBC20 makes CBC20 become disordered [86, 87].

11.4. Maturation of snRNAs. The snRNAs synthesized by RNA polymerase II with m7G cap structures are transported into the cytoplasm in complex with CBP20/80, PHAX (phosphorylated adaptor for RNA export), the CRM1 (export receptor, chromosome region maintenance 1) or exportin 1 and RanGTP (Ras-related nuclear antigen). The snRNPs in the cytoplasm are trimethylated and processed. The mature RNA is reimported into the nucleus in a complex with the trimethyl G cap-specific binding protein snurportin 1 and snRNA binding proteins of Sm RNP and SMN proteins.

Despite immunofluorescent staining of U1 and U2 RNA exclusively in the nucleus [102], biochemical analyses have
Figure 27: Autoradiograph of nucleotides from $[32P]$-labeled U2 RNA 5' fragment [12]. The $[32P]$-labeled U2 RNA 5' fragment (T1 RNase digestion) was treated first with alkaline phosphatase and then with snake venom phosphodiesterase. This mixture of mononucleotide products was separated by electrophoresis followed by chromatography. Approximately equal amounts of pm$_{2,2,7}G$, pAm, pUm, pC, and pG were observed (Table 11).

Figure 28: The susceptibility of 5' cap to pyrophosphatase [12]. The $[32P]$-labeled 5' oligonucleotide obtained from U2 RNA by RNase A was digested with pyrophosphatase and base composition was analyzed by snake venom phosphodiesterase digestion. This digestion released m$_{2,2,7}G$ from the 5' fragment indicating that m$_{2,2,7}G$ is linked by a pyrophosphate linkage.

Figure 29: 2D map of U3 RNA digest [58]. The $[32P]$-labeled U3 RNA was digested with T$_2$ RNase and U$_3$ RNase. It produced two 5' fragments "11A" and "11B".

demonstrated that trimethylation and maturation of some snRNA takes place in the cytoplasm.

The U1 snRNA [103] and U2 snRNA [104] have been shown to be hypermethylated in the cytoplasm in a Sm protein binding dependent manner. The Xenopus laevis U1 RNA, with the m$^7$G cap, has been shown to be hypermethylated in HeLa cell cytoplasmic extracts and Sm binding site in U1 RNA is required [103]. The Tgs1 has been shown to bind to Sm proteins of Sm B and Sm D. The Xenopus laevis U2 RNA with m$^7$G cap has been shown to be hypermethylated into the m$_{2,2,7}G$ cap structure in enucleated xenopus oocytes [104]. In yeast and human HeLa cells, the Tgs1 for U3 RNA is localized in the nucleolar body of the nucleolus and Cajal bodies, respectively [105]. In the absence of Tgs1 or inactive Tgs1 in yeast, m$^7$G capped unprocessed U1 RNA is retained in the nucleolus and splicing becomes cold temperature sensitive. The same enzyme is responsible for the U3 nucleolar RNA hypermethylation [106]. The consensus between yeast and human cells is the presence of a nucleolar body in yeast and Cajal body in HeLa cell. The hypermethylation and processing during maturation take place in the nucleolar body in yeast and Cajal body in HeLa cells [105, 106]. The sequence element “UGAG” (also found in the U3 RNA B box) has been reported as a CAB box (Cajal-body-specific localization signal). U3 RNA trimethylation is somewhat different from other snRNAs. The U3 RNA, which does not have Sm protein binding sites, has been shown to require an intact 3' terminal stem structure for trimethylguanosine cap formation [107].

In HeLa cells, transfected U3 RNA gene products are trimethylated and mature U3 RNA is localized in the nucleolus. Immature U3 RNA, with both m$^7$G and 3' extension of 10–15 nucleotides, is detected in Cajal bodies. The nucleolar localization requires the CAB box, hypermethylation to m$_{2,2,7}G$ cap, and maturation of the 3' end [105]. Unlike U1 RNA and U2 RNA, U3 RNA has been shown to be retained in the nuclear compartment and does not go into the cytoplasm for its trimethylation reaction [105, 106, 108].

12. The Tgs1 (Trimethylguanosine Synthase 1)

12.1. Human Tgs1. The Tgs1, trimethylguanosine synthase in human, protein is 110 kDa and 852 amino acids in chain
length. The gene is located in chromosome 8q11. The mRNA is 3.2 kb in length and produces a 110 kDa protein and ∼65–70 kDa protein that is proteasome process. The long form is in the cytoplasm, and the short isoform has been reported to be localized in the Cajal body within the nucleus. The Tgs1 has S-AdoMet methyltransferase signature motifs of X, I, II (include post 1 motif), III, IV, V, and VI [70, 106, 109, 110].

The human Tgs1 motifs are the following.

- motif X is (a.a.665)DREGWFSVTPEKIAEHI/FA(a.a.682),
- motif I is (a.a.693)VVVDAFCGVGGN(a.a.704),
- motif II is (a.a.714)RVIAIDIPV/IKI(a.a.725) and post 1 motif is VIAID which is responsible for S-AdoMet binding to the enzyme,
- motif III is (a.a.740)KIEFICGDFLLLAS(a.a.753),
- motif IV is (a.a.758/759)VVFLSPIWGPGDYA(a.a.771/772),
- motif V is (a.a.785/786)DGFEIFRLSK(a.a.794/795),
- motif VI is (a.a.798/799)NNIVYFLPRNADI(a.a.810/811),

It was reported that trimethylation catalytic activity is located in the C-terminal region (amino acids 631–852) and this region contains the S-AdoMet-dependent methyltransferase motifs. The tryptophan in motif 4 is involved in π stacking with m’G guanosine of the substrate. The motif 1 and post 1 motif are reported to interact with S-AdoMet. [110]. The C-terminal domain is localized in the Cajal body and binds to C/D-snoRNA- and H/ACA-snoRNA-associated proteins such as fibrillarin, Nop56, as well as dyskerin [110].

The N-terminal portion of the molecule (amino acids 1–477) has been reported to contain GXGXGXXI, a K-homology domain for RNA binding, and a motif for SmB and SmD1 binding. The Tgs1 has also been shown to interact with PRIP (proliferator-activated receptor-interacting protein), and the N-terminal portion (amino acids 1–384) of Tgs1 has been shown to have stimulatory effects on transcription of PPARγ and RXRα [109, 110].

The human Tgs1 (618–853) has been crystallized for structural analysis. The one monomer consists of 11 α-helices and 7 β-strands. It is composed of 2 domains, the core domain (Glu675–Asp844) and N-terminal extension (Leu34–Ser671) connected by 3 amino acids—Val672, Thr673, and Ser674. The core domain consists of 7-β-strands in topology of β6↑β7↓β5↑β4↑β1↑β2↑β3↑ with a classical class 1 methyltransferase fold resembling the Rossmann-fold AdoMet-dependent methyltransferase superfamily [90].

The N-terminal α-helices form a separate small globular subdomain involved in recognition and binding of both substrates. The residues Glu667 and Phe670 in motif X as well as Pro765, Trp 766, and Pro769 in motif IV are in proximity permitting the top of their binding clefts to be close together. Tryptophan 766 and m’G are stacked in a coplanar manner with a 3.2 Å distance providing a tight π-π interaction between them (Figure 34).

The catalytic mechanism of methylation is by an $S_{n2}$ substitution reaction. The N2 of m’G does the nucleophilic attack on an activated methyl group of the AdoMet (Figure 35).

Dimethylation is not processive. After formation of m3,2,7G both products (m3,2,7G and AdoHcy) dissociate from the enzyme. Tgs1 can use m2,7G as a substrate, and newly bound AdoMet can methylate at the same position by the same mechanism to form the m3,2,7G cap structure.
in the GDP (cap I, m$^{32,2,7}$GpppAmA) region and “11A” (cap II, standards of GMP, GDP, and GTP. Component “11B” was eluted phosphatase and chromatographed on DEAE-Sephadex A-25 with amino acid protein while dORF is 538 bp from the 5$'\endgroup$ transcription start site and has coding capacity for a 178 ORFs (open reading frames). The uORF is 80 bp from the and 2,600-nucleotide long with upper and downstream U4 snRNAs. The mRNA for the protein Tgs1 is polycistronic trimethylguanosine cap formation activity for both U2 and (Drosophila TAT-like) has been reported to exhibit In Drosophila melanogaster, DTL $12.2. \text{Drosophila Tgs1}$. In Drosophila melanogaster, DTL (Drosophila TAT-like) has been reported to exhibit trimethylguanosine cap formation activity for both U2 and U4 snRNAs. The mRNA for the protein Tgs1 is polycistronic and 2,600-nucleotide long with upper and downstream ORFs (open reading frames). The uORF is 80 bp from the transcription start site and has coding capacity for a 178 amino acid protein while dORF is 538 bp from the 5$'\endgroup$ end and produces a 60 kDa protein (491 amino acids). The two cistrons are overlapped by 76 bp. Mutational analysis indicates that both the uORF and dORF regions are required for viability. The putative product of uORF contains periodic Leu residues, but there is no evidence that this region is translated at any time during Drosophila development. The protein from dORF contains an Arg-rich motif KKKR-RQRQI similar to the RNA binding motif RKKRRQRRR in HIV TAT. This protein is localized in the nucleus and responsible for trimethylation of U2 and U4 snRNAs [111].

12.3. Yeast Tgs1. In yeast S. cerevisiae, Tgs1 is in the nucleolus and U3 RNA is also in the nucleolus. In the absence of Tgs1, the pre-U3 RNA was found within the nucleolar body and U1 RNA was retained in the nucleolus. S. cerevisiae, S. pombe, and Giardia lamblia Tgs1 can methylate m$^7$GTP, m$^7$GDP, and m$^7$GpppA as substrates without preassembly of snRNP containing Sm proteins. The Tgs1 of S pombe is 239-amino-acid long and m$^7$G is the pre-requisite for this reaction [112].

12.4. The G. lamblia Tgs1 and Tgs2. The lamblia has 2 enzymes, Tgs1 and Tgs2. Tgs1 is not a processive enzyme but distributive and produces m$^{32,2,7}$G in excess of AdoMet and enzyme. However, Tgs2 produces only m$^{2,7}$G, and some G. lamblia RNAs contain dimethylG caps. The G lamblia Tgs1 has 300 amino acids and Tgs2 is 258 amino acids long. They all have landmark motifs for Ado-Met-dependent methyltransferase motifs [113].

13. Parasite Capping Enzyme (Trypanosoma brucei)

The parasite Trypanosoma brucei SL RNA (splicing leader) has the biggest 5$'\endgroup$ oligonucleotide, type IV, of m$^7$Gppp$m^6$AmpCmpAmpCmp$m^3$UmpAp [114, 115]. Enzymes involved in the synthesis of this cap structure are TbCgm1, TbCet1, TbMTr1(cap1 2’OMTase), TbMTr2/TbCom1/TbMT48(cap2 2’OMTase), TbMTr3/TbMT57(cap3 2’OMTase). However, m$^6$A and m$^3$U methylating enzymes have not been identified as yet [115].

13.1. TbCgm1 (T. brucei Cap Guanylyltransferase Methyltransferase 1). There exist two enzyme systems for 5$'\endgroup$ cap formation. The first is the system composed of separate independent enzymes which are TbCet1 (Trypanosoma brucei triphosphatase, 253 amino acids), TbCe1 (Trypanosoma brucei guanylyltransferase, 586 amino acids), and TbCmt1 (Trypanosoma brucei m$^7$G Cap methyltransferase 1, 324 amino acids). The second is a set of fused enzymes possessing dual activities. It is TbCgm1 (Trypanosoma brucei cap guanylyltransferase and methyltransferase 1) that has 1050 amino acids [116] with dual activities of guanylyltransferase and guanine N-7 methyltransferase [117]. The TbCe1 guanylyltransferase has 250 amino acids at its N-terminal region which is not found in fungal or metazoan guanylyltransferase and has homology with the phosphate binding loop found in ATP- and GTP-binding proteins [118]. Silencing TbCe1 and TbCmt1 had no effect on parasite growth or SL RNA capping, but TbCgm1 was essential for parasite growth and silencing TbCgm1 increased the amount of uncapped SL RNA. The protein TbCgm1 has guanylyltransferase activity in N-terminal 1–567 amino acids and methyltransferase activity in C-terminal 717–1050 amino acids. The N-terminal guanylyltransferase portion contains 6 colinear guanylyltransferase motifs: I(KADGTR), III(FVDAELM), IIIa(LIGCFDVFRYVI), IV(DGFIF), V(QLXWKPSMLVD), and VI(WSIERLRNDK). The C-terminal methyltransferase portion contains regions homologous to m$^7$G methyltransferase from T. cruzi and L. major [117].

13.2. Cap Methylating Enzymes: TbMTr1, TbMTr2(TbCom1/TbMT48), and TbMTr3(TbMT57). They contain a K$^{95}$-D$^{207}$-K$^{248}$-E$^{285}$ tetrad critical for AdoMet-dependent
methyltransferase and can convert cap type 0 of *Trypanosoma* SL RNA and U1 snRNA into type 1 cap [115]. The KDKE mediates $S_{\text{m}}$ type transfer of methyl groups that involve 2'-OH deprotonation. The U1 snRNA 2'-O-methylation takes place before Sm protein binding to the RNA and it is prerequisite for the dimethylation at the N2 position to make $m\_3\_2\_7$GpppAm cap structures. Other $m\_3\_2\_7$G cap-containing snRNAs such as U2, U-snRNA B (U3 snRNA homolog), and U4 snRNAs were reported to be synthesized by RNA polymerase III in *Trypanosomes*.

The TbMTr2 and TbMTr3 are responsible for second and third nucleotides 2'-O-methylation. The enzymes that perform $m\_6\_6\_A$, $m\_3\_U$ base methylations, and fourth nucleotide 2'-O-methylating enzymes are not known yet.
Figure 34: Crystal structure of hTgs1 [90]. The crystal structure of hTgs1 with substrate m7GTP and AdoHcy (at the site for AdoMet). The m7G is stacked between tryptophan 766 and serine 671 [90]. (a) Relative orientation of substrates m7GTP and AdoHcy at binding pockets. (b) Detailed view of the binding pocket for m7GTP (shown W766 and S671). (c) Detailed view of the binding pocket for AdoHcy.

Figure 35: The active site of hTgs1 [90]. Proposed mechanism of methyltransferase activity of hTgs1 is shown. The AdoMet methyl group is in close proximity to N2 position of m7GTP. The prerequisite as a substrate for hTgs1 is m7G moiety.

14. Transport of Mature RNAs

The snurportin1 is a specific trimethyl G cap binding protein with an importin β binding site at its N-terminus (amino acids 1–65) and trimethyl G cap binding site at amino acids 95–300 forming a cap binding pocket. This protein has more resemblance to mRNA guanylyltransferase. The snurportin 1 binds the trimethyl G cap forming π-stacking with tryptophan 276 and the penultimate purine nucleotide G (Figure 36). The tryptophan 107 is in close proximity to dimethylamine of N2 G suggesting a cation-π interaction and has a role in discriminating between m7G cap and m1,2,2,7G cap [91].

15. Tgs1 Interacting Proteins

Genetic and biochemical analysis of Tgs1 interacting proteins reveals a wide range of proteins involved in RNA metabolism.
Figure 36: The comparison between \( m_3^{2,2,7}G \) binding mode to snurportin1 and \( m_7^G \) cap binding mode to CBP 20, eIF4E and VP39 [91]. The \( m_3^{2,2,7}G \) is stacked between tryptophan 107 and penultimate nucleotide G of cap core \( m_3^{2,2,7}GpppG \).

Figure 37: The sequence of 4.5S RNA I (Novikoff hepatoma cell nuclei).

It interacts with proteins in the transcriptional apparatus, RNA end processing and decay, spliceosomal assembly and RNA modifying factors (Table 12).

Structurally, it is distinct from the \( m_7^G \) cap, and the specificity of binding proteins may determine the precision of its functional role in the RNP complex. The \( m_3^{2,2,7}G \) cap structures are present only in nuclear snRNAs and snoRNAs which confer the function within the nucleus in transcription, splicing, modification, processing, and maturation of different RNA species.

16. Conclusion

16.1. General Consideration. In the present postgenomic era, study of the structure and function of noncoding RNAs is supremely important. It is estimated that ncRNAs are probably involved in all aspects of cell metabolism. Therefore, RNA-based information will contribute greatly to understanding various cell metabolisms. In the process of exploring ncRNAs, there may be many surprises awaiting us. They may include

(1) new species of RNA,
(2) new mechanism of RNA processing,
(3) new mechanism of transcription,
(4) new disease caused by RNAs with pathogenic sequences,
(5) new function for ncRNA.

16.2. The Problem of Unknown Modified Nucleotides. In the process of oligonucleotides cataloging, it is natural that an examination of base composition will reveal modified nucleotides or nucleosides in addition to unmodified standard nucleotides or nucleosides. In routine work, identification of modifications can be readily made by
two-dimensional paper chromatography for nucleotides or thin layer chromatography for nucleosides. However, there may be an occasion where chromatographic identification is not sufficient. Of course, it is best to have collaboration with outside specialists. For the sake of structural microanalysis, it is highly recommendable to determine molecular weight of the unknown nucleotide or nucleoside by mass spectrometry [119]. The required quantity is approximately 5 μg/nt where chromatographic identification of isotopically labeled sample requires 0.5 μg/nt. A difficulty may be confronted with purine bases that are fused to an imidazole ring (Queuosine) which is not suited for mass spectrometry. It is convenient to probe chemical complexity based on mass. The detailed analysis may require an unpredictably large amount of samples. There are 135 modified nucleosides listed, among which 6 nucleosides are not thoroughly identified [1].

16.3. Significance of Sequence Work. Past sequence work has permeated numerous significant areas of research providing a better understanding of cellular metabolism. The information obtained thus far is RNA-based information which is not seen in DNA, proteins, and others. As sequence work continues to make enormous progress, the postgenomic era will shape the direction of research in the area of molecular mechanisms of RNA metabolism. They are briefly as follows.

In RNA maturation, knowledge of structural modifications is necessary to discern between various mechanistic options. For example, there are two molecular mechanisms mediated by catalysis. One is mediated by RNA enzymes (snRNAs and snoRNAs) involved in splicing of pre-mRNA and processing of pre-rRNA. The other is protein enzymes involved in 5' cap formation. Currently, the higher order structural analysis is in progress. There is a need to elucidate the details of molecular mechanisms.

Along with the study of splicing physiology, splicing pathology is making significant progress. Aberrant modifications can generate disease causing alterations in structure. The aberrations cause problems in reading both genetic codes and splicing codes. Studying the regulation of alternative splicing will clarify the selective rules in intron removal and pathogenic rules in splicing code. From these studies, corrective strategy will evolve. The present sequence work is engaged in definition of ncRNAs diversity and their functional roles [120]. Since it is suggested that ncRNAs are involved in all aspects of regulations in cell metabolism, there may be opportunities to study various paths in cell metabolism, not limited to transcriptional and posttranscriptional events. It is this gigantic task, to reevaluate the genomic work, that holds excitement and promise.

Abbreviations Used in Table 4

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>miRNA</td>
<td>MicroRNA (imperfect base pairing)</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA (perfect base pairing)</td>
</tr>
<tr>
<td>tasiRNA</td>
<td>Transacting small interfering RNA</td>
</tr>
<tr>
<td>natsiRNA</td>
<td>Natural antisense transcribed small interfering RNA</td>
</tr>
<tr>
<td>piRNA</td>
<td>PIWI interacting RNA (RNA precipitated by PIWI protein antibody)</td>
</tr>
<tr>
<td>rasi RNA/pitRNA</td>
<td>Repeat-associated small interfering RNA/pi-target RNA</td>
</tr>
<tr>
<td>PARs</td>
<td>promoter associated RNAs</td>
</tr>
<tr>
<td>PROMTs</td>
<td>Promoter upstream transcripts (sense and antisense transcript)</td>
</tr>
<tr>
<td>PASRs</td>
<td>Promoter-associated small RNAs</td>
</tr>
<tr>
<td>TSSa-RNAs</td>
<td>Transcription-start-site-associated RNAs</td>
</tr>
<tr>
<td>tRNAs</td>
<td>Transcription initiation RNAs</td>
</tr>
<tr>
<td>MSY-RNA</td>
<td>MSY2-associated RNAs (MSY; Y chromosome male-specific protein)</td>
</tr>
<tr>
<td>snoRNA</td>
<td>Small nucleolar RNA (C/D box RNA, H/ACA RNA)</td>
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<td>sdRNA</td>
<td>sno-derived RNAs</td>
</tr>
<tr>
<td>moRNA</td>
<td>MicroRNA-offset RNAs</td>
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<tr>
<td>tel-sRNA</td>
<td>Telomere small RNAs</td>
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<tr>
<td>crasiRNA</td>
<td>Centrosome-associated small interfering RNAs</td>
</tr>
<tr>
<td>hsRNA</td>
<td>Heterochromatin small RNA or hairpin small RNA</td>
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<tr>
<td>scaRNAs</td>
<td>Small Cajal-body-associated RNAs</td>
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<tr>
<td>Y RNAs</td>
<td>Cytoplasmic small RNA Y1, Y3, Y4, and Y5</td>
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<tr>
<td>tRNA-derived RNAs</td>
<td>Small RNA processed from tRNA by RNase (angiogenin)</td>
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<tr>
<td>Alu/SINE RNA</td>
<td>Alu restriction enzyme cleaved repeat gene transcript/short interspersed nucleotide element RNA.</td>
</tr>
<tr>
<td>Lnc RNA</td>
<td>Long Noncoding RNA (~0.5 to 100 kb)</td>
</tr>
</tbody>
</table>

(1) Specific Long Noncoding RNA

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TR/TERC</td>
<td>Telomerase RNA/telomerase RNA component</td>
</tr>
<tr>
<td>NEAT RNAs</td>
<td>Nuclear enriched abundant transcript 1 RNAs</td>
</tr>
<tr>
<td>NEAT1v-1</td>
<td>NEAT1 variant 1</td>
</tr>
<tr>
<td>NEAT1v-2</td>
<td>NEAT1 variant 2</td>
</tr>
<tr>
<td>NEAT2/MALAT1</td>
<td>Metastasis associated in lung adenocarcinoma transcript 1</td>
</tr>
<tr>
<td>PINC RNA</td>
<td>Pregnancy-induced noncoding RNA</td>
</tr>
<tr>
<td>DD3/PCA3</td>
<td>Prostate-cancer-associated RNA 3</td>
</tr>
<tr>
<td>PCGEM1</td>
<td>Prostate cancer gene expression marker 1</td>
</tr>
<tr>
<td>SPRY4-1T1</td>
<td>Sprouty homolog 4 gene transcript 1 (melanoma specific).</td>
</tr>
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</table>
(2) Imprinting-Associated lncRNAs

- xiRNAs: X chromosome inactivating RNAs
- Xist: X chromosome inactivating sense RNA
- Tsix: Antisense transcript of Xist
- RepA: Repeat A RNA

- AIR RNA: Igf2r imprinting region RNA
- H19: Igf2 imprinting region RNA
- KCNQ1ot1: Antisense RNA from intron 10 of Kcng1 gene imprinting region.

(3) Regulatory lncRNAs

- HOTAIR: Homeogene inactivating RNA
- BORG: BMP/OP-responsive-gene-associated RNA
- CTN RNA: Cationic amino acid transporter protein coding region RNA
- ANRIL RNA: Antisense noncoding RNA in INK4 locus.

(4) Gene-Recombination-Associated lncRNA

- LINE: long interspersed nucleotide element
- CSR-RNA: Immunoglobulin class switch recombination region RNA.

(5) Satellite DNA Transcripts.

Abbreviations for Table 12

- Mud: Mutant U1 die
- RES complex: Heterotrimeric RNA retention and splicing complex composed of Bud13, Ist3/Snu17, and Pml1
- Swt21: Synthetic with Tgs1 number 21
- TRAMP complex (Trf4, Air2, Mtr4p polyadenylation complex): Interacts with exosome in the nucleus and involved in 3' end processing of rRNA, snoRNA, and U1, U4, and U5 snRNA;
  - Trf4 or Trf5: poly(A) polymerase(PAP);
  - Mtr4: RNA helicase;
  - Air1 or Air2: Zinc knuckle protein
- Cbf5(YLR175W): Centromere binding factor;
  - Pseudouridine synthase catalytic subunit of box H/ACA snoRNP complex
- PIMT: PRIP-interacting protein with methyltransferase domain.
- PIMT is a Tgs1 (trimethylguanosine synthase 1) cloned from human liver cDNA library
- PRIP: PPAR interacting protein
- PPAR: Peroxisome proliferator-activated receptor
- PBP: PPAR binding protein.

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References


Research Article

Site-Selective Artificial Ribonucleases: Oligonucleotide Conjugates Containing Multiple Imidazole Residues in the Catalytic Domain

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Design of site-selective artificial ribonucleases (aRNases) is one of the most challenging tasks in RNA targeting. Here, we designed and studied oligonucleotide-based aRNases containing multiple imidazole residues in the catalytic part and systematically varied the structure of cleaving constructs. We demonstrated that the ribonuclease activity of the conjugates is strongly affected by the number of imidazole residues in the catalytic part, the length of a linker between the catalytic imidazole groups of the construct and the oligonucleotide, and the type of anchor group, connecting linker structure and the oligonucleotide. Molecular modeling of the most active aRNases showed that preferable orientation(s) of cleaving constructs strongly depend on the structure of the anchor group and length of the linker. The inclusion of deoxyribothymidine anchor group significantly reduced the probability of cleaving groups to locate near the cleavage site, presumably due to a stacking interaction with the neighbouring nucleotide residue. Altogether the obtained results show that dynamics factors play an important role in site-specific RNA cleavage. Remarkably high cleavage activity was displayed by the conjugates with the most flexible and extended cleaving construct, which presumably provides a better opportunity for imidazole residues to be correctly positioned in the vicinity of scissile phosphodiester bond.

1. Introduction

The idea of site-selective artificial ribonucleases which are capable of cleaving any particular RNA sequence in vitro and in vivo, is a very attractive approach as, apart from being useful tools in molecular biology, these chemical ribonucleases are anticipated to be helpful for target validation, and even for the development of potential antiviral or anticancer therapeutics. The idealized site-selective artificial ribonuclease would be a compound that is easily synthesized, chemically stable, targeted to any chosen RNA sequence, and highly efficient in cleaving of the phosphodiester bonds. The oligonucleotide-based artificial ribonucleases seem to meet the majority of these criteria due to the fact that they can be directed to almost any desired RNA region in a site specific-manner by controlling the sequence of the oligonucleotide recognition part.

Virtually all types of the reactive groups, which are known to catalyze RNA scission, have been exploited for design and preparation of artificial ribonucleases [1–3]. Conjugates of oligonucleotides and RNA cleaving groups can be synthesized using two main approaches: incorporation of a catalytic structure in the oligonucleotide in the course of standard synthesis or by postsynthetic derivatization of an unprotected oligonucleotide [4]. Several alternative procedures for each strategy are available. The first approach often involves the automated oligonucleotide synthesis, when phosphoramidites of suitably protected nucleosides and
catalytic groups are used as building blocks. Monomeric building blocks containing various metal complexes [5–8] or imidazole-based structures [9–16] have been previously proposed for the synthesis of the oligonucleotide-based artificial ribonucleases. The successive synthesis of oligonucleotide and catalytic structures of conjugates on the same solid-phase support is a promising variation of this approach [17–19]. However, this approach is inappropriate for the majority of the potential users of the conjugates due to the necessity to synthesize the unique phosphoramidite derivatives of the corresponding catalytic structures in each particular case, which creates the problems associated with the choice of appropriate protection groups and the deprotection procedure.

Various RNA-cleaving catalysts have been tethered to the 5′-terminus of oligonucleotides. However, the available synthetic methods, except for a few examples [20], allow the incorporation of only a single functional moiety per phosphoramidite unit. Generally, 5′-tethering can be implemented in two ways either by direct incorporation of a suitably protected and activated constructs into the 5′-terminus at the end of standard solid phase synthesis or by postsynthetic derivatization. In the former case, synthesis of highly modified oligonucleotides may become somewhat problematic because increase in a number of incorporations is expected to substantially decrease the overall yield of the conjugated oligonucleotides. Alternatively, a precursor molecule can first be formed by reacting the 5′-OH group of an assembled oligonucleotide with a heterobifunctional reagent (first modifier) bearing both the phosphoramidite moiety and a reactive group. The reactive group of the precursor oligonucleotide is then postsynthetically derivatized with an appropriate functional additive (second modifier) [21, 22]. This precursor strategy has the obvious advantage that the same parent compound can be used to synthesize a number of differently tethered products. The first generation of oligonucleotide-based artificial ribonucleases, which was prepared using the precursor technique, has recently been proposed for the synthesis of the oligonucleotide-based artificial ribonucleases. The successive synthesis of oligonucleotide and catalytic structures of conjugates on the same solid-phase support is a promising variation of this approach [17–19]. However, this approach is inappropriate for the majority of the potential users of the conjugates due to the necessity to synthesize the unique phosphoramidite derivatives of the corresponding catalytic structures in each particular case, which creates the problems associated with the choice of appropriate protection groups and the deprotection procedure.

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However, the cleavage activity of these artificial ribonuclease so far has been inferior to the natural counterparts in terms of targeting and/or cleavage efficiency. We anticipate that the high level of efficiency of RNA cleavage by these oligonucleotide-based artificial ribonucleases can only be achieved if the optimal arrangement of all groups involved in the binding and catalysis is accomplished. It might be possible to improve on this by iterative design of the binding and cleavage domains by taking into account the optimal structures of the cleaving groups and their mutual spatial orientations against the RNA target. The structural organization of artificial ribonucleases and the dynamic behavior of their cleaving constructs seem to be the key factors contributing to the cleavage activity of these compounds.

Several years ago, we reported a new type of oligonucleotide-based aRNases containing bisimidazole cleaving constructs, which were prepared by precursor technique. It was demonstrated that these highly active bis-imidazole cleaving constructs were extremely flexible and the cleavage of the phosphodiester bond seemed to be a random event, which happened with the high efficiency every time, when these catalytic groups approached the scissile phosphodiester bond [23, 24]. In the present paper, we use the developed precursor approach to synthesize the scaffolds of aRNases with the systematically varied structure of the cleaving constructs bearing multiple imidazole residues in the catalytic part. We demonstrate here that some of these conjugates exhibit remarkably high cleavage activity within the tRNA⁰⁰⁰⁰⁰ conjugate hybrid and identify possible location(s) of cleaving group(s) relative to the scissile ⁰⁰⁰⁰⁰⁰⁰⁰ site. We also show, here, that the cleaving activity of the conjugates is governed by the intrinsic properties of the catalytic constructs, but not the detailed structure of the DNA:RNA hybrid, which was the same in all experiments.

2. Materials and Methods

2.1. General Chemicals. All buffers used in the experiments were prepared using milliQ water, contained 0.1 mM EDTA, and were filtered through filters with pore size 0.22 μm (Milipore). [5′-32P]-pCp was from Biosan Co., Russia. T4 RNA ligase was purchased from Fermentas (Lithuania). Oligonucleotides TGTTGCGAATTCTG (A) and GATCGAGCAAGCCCT (B) were synthesized on ASM-700 DNA Synthesizer (Biosset, Russia) by standard solid-phase phosphoramidite procedure. Yeast tRNA³⁴⁶ was a generous gift from Dr. G. Keith (Institut de Biologie Moléculaire et Cellulaire du CNRS, Strasbourg, France). Methoxyoxalamido (MOX) modifiers M used for conjugate synthesis were prepared as described in [21, 24].

2.2. Synthesis of Oligonucleotide Conjugates B-Im(n) Type 1, Type 2, Type 3, and Type 4. Deoxyribonucleotides B (GATCGAGCAAGCCCT) was synthesized using standard solid-phase phosphoramidite chemistry, except that the dC⁰⁰⁰⁰⁰⁰⁰⁰⁰-phosphoramidite was replaced with the dC⁰⁰⁰⁰⁰⁰⁰⁰⁰-phosphoramidite. At the end of the automatic synthesis, the core 17-mer was coupled with one of the modifiers M (Type 1, Type 2, Type 3, or Type 4; see Table 1) in 0.2 M in acetonitrile for 15 min. The prepared oligonucleotide MOX precursors were then functionalized with 2 M solution of histamine in dimethylformamide for 3 hrs at 20°C at constant shaking followed by deprotection with ammonia to produce the oligonucleotide conjugates containing multiple-imidazole catalytic structures. The conjugates were purified by electrophoresis in 16% polyacrylamide/8 M urea gel under denaturing conditions. The purified conjugates were analyzed by ESI and/or MALDI MS (calculated and experimental values of molecular masses are listed in Table 1). In the cases when MALDI-MS analysis did not produce the expected mass spectra due to the conjugate fragmentation upon analysis, the conjugates were analyzed by electrophoresis under denaturing conditions (Figure 1(c)) and by HPLC (primary data not shown). Conjugates were designated as B-Im(N/M), where B-Im corresponds to the oligonucleotide B and shows that Im are in catalytic part, N is the number of
Figure 1: Continued.
**Figure 1:** Schematic representation of the catalytic structures of the site-selective artificial ribonucleases B-Im(N/m) and analysis of the conjugates. (a) Anchor groups used for attachment of dendrimeric RNA-cleaving constructs to oligonucleotide B 5'-phosphate. (b) Schematic representation of conjugates bearing four and eight imidazole residues and anchor groups of Type 1, Type 2, and Type 3, obtained using precursor technology. Mass spectrometry data: 1: calculated mass 5523.30, found mass 5523.73; 2: 5566.80/5566.39; 3: 5692.89/5692.03; 4a: 6838.95/6838.65; 5: 6423.63/6425.03. (c) Analysis of the conjugates B-Im(N/m) by 12% polyacrylamide/8 M urea gel electrophoresis. 0.1 A_260 unit of each conjugate was applied on the gel; the conjugates were visualized in the gel by staining with Stains-All. Numbers on the top of the gel correspond to numeration of the conjugates in the Table 1 and Figure 1(b) and Supplementary Material Figure 1.
imidazole residues (from 2 to 32), and m corresponds to the type of anchor group (1, 2, 3, or 4; see Figure 1(a)).

2.3. Cleavage of tRNA\textsuperscript{Phe} with Oligonucleotide Conjugates. [3',\textsuperscript{32}P]-tRNA\textsuperscript{Phe} was prepared and purified as described in [24]. 3'-end labeled tRNA was dissolved in water and stored at −20°C. Specific radioactivity of the [\textsuperscript{32}P]-tRNA\textsuperscript{Phe} was 5 × 10\textsuperscript{6} cpm/pmole.

Standard reaction mixture (10 μL) contained imidazole buffer (50 mM imidazole buffer, pH 7.0, 200 mM KCl, 0.1 mM EDTA, 100 μg/mL total tRNA from \textit{Escherichia coli} as carrier), 5 × 10\textsuperscript{−7} M [3',\textsuperscript{32}P]-tRNA\textsuperscript{Phe} and one of oligonucleotide conjugates \textbf{B-Im(N/m)} at concentrations ranging from 5 × 10\textsuperscript{−7} to 5 × 10\textsuperscript{−4} M (as indicated in the legends in the figures). Reactions were carried out at 37°C and were quenched by precipitation of tRNA and tRNA fragments with 150 μL of 2% lithium perchlorate solution in acetone. RNA was collected by centrifugation and dissolved in loading buffer (6 M urea, 0.025% bromophenol blue, 0.025% xylene cyanol). tRNA cleavage products were analyzed by electrophoresis in 12% polyacrylamide/8 M urea gel. To identify cleavage sites, an imidazole ladder [24] and an A- \textit{G} ladder produced by partial tRNA cleavage with RNase T1 [25] were run in parallel. To obtain quantitative data, the gel was dried and analyzed using Molecular Imager FX (Bio-Rad). The total extent of RNA cleavage was determined as a ratio of radioactivity found in the tRNA fragments to total radioactivity applied on the gel lane.

To measure the kinetics of the tRNA cleavage with the oligonucleotide conjugates \textbf{B-Im(N/m)}, the reaction mixture of 100 μL was prepared containing imidazole buffer, 5 × 10\textsuperscript{−7} M 3'-[\textsuperscript{32}P]-tRNA\textsuperscript{Phe} and one of the oligonucleotide conjugates \textbf{B-Im(N/m)} at different concentrations (from 5 × 10\textsuperscript{−7} to 5 × 10\textsuperscript{−4} M). Reaction was performed at 37°C. At indicated time intervals, an aliquot (10 μL) of the reaction mixture was withdrawn and frozen for lately assay. Reaction aliquots were defrosted and immediately precipitated with 150 μL 2% lithium perchlorate in acetone. After that, the reaction mixtures were assayed as described above.

In some experiments, imidazole buffer was replaced by HEPES-buffer (50 mM HEPES-KOH, pH 7.0; 0.2 M KCl, 0.1 mM EDTA, 100 μg/mL RNA-carrier), or Ca\textsubscript{2+} buffer (50 mM Ca\textsubscript{2+} acyldiol-acid-KOH, pH 7.0; 0.2 M KCl, 0.1 mM EDTA, 100 μg/mL RNA-carrier) or Tris-HCl-buffer (50 mM Tris-HCl, pH 7.0; 0.2 M KCl, 0.1 mM EDTA, 100 μg/mL RNA-carrier). The experiments where imidazole buffer was replaced by other buffers are indicated in text and figure legends.

2.4. Inhibition of Site-Selective tRNA\textsuperscript{Phe} Cleavage by Oligonucleotide Competitor. In the competition experiments, to a reaction mixtures (10 μL) containing imidazole buffer, 5 × 10\textsuperscript{−7} M 3'-[\textsuperscript{32}P]-tRNA\textsuperscript{Phe} oligonucleotide A (complementary to sequence 62-76 of the tRNA\textsuperscript{Phe}) or oligonucleotide B was added up to concentration 5 × 10\textsuperscript{−5} M. The mixtures were incubated for 10 min at 37°C, then one of oligonucleotide conjugates \textbf{B-Im(N/m)} at concentration 5 × 10\textsuperscript{−6} M was added and the mixtures were incubated at 37°C for different times (from 30 min to 2 h). Reaction was quenched and analyzed as described above.

2.5. Gel-Mobility Shift Assay of Hybridization of Oligonucleotide B and Conjugates B-Im(4/2) to tRNA\textsuperscript{Phe}. A gel-mobility shift assay of tRNA\textsuperscript{Phe} binding with oligonucleotide B and conjugates B-Im(4/2) was performed as follows. [3',\textsuperscript{32}P]-tRNA (5 × 10\textsuperscript{−7} M) was incubated at 37°C during 30 min in reaction mixture (10 μL) containing hybridization buffer (50 mM Tris-HCl, pH 7.0, 200 mM KCl, 0.1 mM EDTA) and oligonucleotide B or conjugate B-Im(4/2) ranging in concentration from 1 × 10\textsuperscript{−7} to 5 × 10\textsuperscript{−6} M. After incubation, 8 μL of loading buffer (50% glycerol, 0.025% bromophenol blue, 0.025 xylene cyanol) was added to each probe and probes were immediately applied onto running native 10% PAGE with 100 mM Tris-borate, pH 8.3 as running buffer preequilibrated at 4°C for 3 h. Electrophoresis was performed at 4°C for 5 h at 450 V. To obtain quantitative data, the gel was dried and analyzed using Molecular Imager FX (Bio-Rad).

2.6. Molecular Modelling. Molecular modelling was performed using SYBYL 6.6 (TRIPOS Inc.) on a Silicon Graphics O2 workstation as described in [24]. All calculations (MM/MD) were carried out in \textit{vacuo} using Kollman-All force field parameters and a distance-dependent dielectric constant equal to 4. The building of the model molecules (see Figure 1) was carried out in three stages. First, an A-like DNA:RNA hybrid was built using BIOPOLYMER/SYBYL 6.6. Second, the catalytic fragments \textbf{Im(4/1)}, \textbf{Im(4/2a)}, and \textbf{Im(4/2b)} were built using SKETCH/SYBYL 6.6. Charge distributions for these constructs were calculated using a semi-empirical MO program, MOPAC, after geometric optimization. In the last step, each the corresponding cleaving fragment was attached to the DNA:RNA hybrid via the terminal 5'-phosphate group of the G1 nucleotide residue.

For each molecule, several different starting structures were created, differing in the conformation and location of the cleaving group, ranging from "in" to "out" extreme positions relative to the DNA:RNA hybrid. The starting structures of each molecule were subjected to minimization to a gradient convergence of 0.05 Kcal/mol, considering the DNA:RNA hybrid as a solid body. Each starting structure was individually subjected to a 12 ps MD run using simulated annealing as follows. The system was heated at 600 K during 2 ps followed by a gradual cooling down to 100 K over 10 ps. A final minimization to convergence criteria was performed resulting in the respective final conformations for each type of molecule. S and F symbols before the conjugate name indicate the starting and final conformation, respectively.

"Active conformations" were obtained for each model molecule (O1-Im4/1, O2-Im4/1 O1-Im4/2a, O2-Im4/2a, O1-Im4/2b, and O2-Im4/2b, where Type1 shows the anchor group 1) using a similar simulating annealing protocol. In these molecules, the cleaving groups were deliberately located in the most favorable position for successful cleavage at the C\textsubscript{63}-A\textsubscript{64} site. Namely, the distance between N1 of one of the imidazole residues and 2' OH group of C\textsubscript{63} was ~3 Å and the distance between N1 of the other imidazole residue and
3. Results and Discussion

3.1. Design of Oligonucleotide-Based Artificial Ribonucleases.

The RNA cleaving oligonucleotide conjugates have been prepared in two-step procedure described in [21, 22]. In the first step, the precursor 17-mer GATCGAACACAGGACCT (B) was built up with multiple methoxyoxalamido (MOX) modifiers of different type (see Supplementary Material available online at doi:10.4061/2011/748632 for the structures of all conjugates synthesized in this study). In the second step, the prepared oligonucleotide-MOX precursors were functionalized with histamine to yield after deprotection step the oligonucleotide-MOX precursors were function-

Nonnucleotide insertion in oligonucleotide. Type 4 anchor groups of Types 1 and 2 were incorporated also into the linker structure mimicking phosphodiester bond and used as a rule as nonnucleotide insertion in oligonucleotide. Type 4 anchor group was used in the conjugates 10–13 to increase the number of branches, bearing imidazole residues. For this purpose and to increase the linker length and flexibility, the anchor groups of Types 1 and 2 were incorporated also into the linker structures (see Figure 1-SM, conjugates 6–9 and 11–13). Moreover, the length and structure of linkers, connecting imidazole residues and anchor, were distinguished sufficiently among the compounds (Table 1 and Figure 1-SM). Conjugates containing equal number of imidazole residues, for example, B-Im(4/m) or B-Im(8/m), differ not only in anchor groups, but also in a linker length. The linker of conjugate B-Im(4/3) was the shortest one. In this conjugate, four imidazole residues were placed nonsymmetrically in respect to the phosphate groups forming the anchor, which connected the oligonucleotide B and the cleaving construct.

The identity of the conjugates was confirmed by mass-spectrometry; (see Figure 1). In addition, the purity of the conjugates was confirmed by electrophoresis in 12% PAAM/8 M urea gel (Figure 1(c)). It is seen that the purity of the conjugates is close to 95%. Only in the case of conjugates 2,

Table 1: Oligonucleotide-based artificial ribonucleases bearing multiple imidazole groups in the catalytic part: structure of conjugates and efficiencies of site-specific cleavage of yeast tRNA\textsuperscript{Phe}.

<table>
<thead>
<tr>
<th>Conjugate(1)</th>
<th>Number of Im groups</th>
<th>Anchor group(2)</th>
<th>Linker length(3)</th>
<th>Reaction rate, %/1 h(4)</th>
<th>Reaction 1/2(5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 B-Im(2/1)</td>
<td>2</td>
<td>1/2</td>
<td>21</td>
<td>24.8</td>
<td>2 h 15(^*)</td>
</tr>
<tr>
<td>2 B-Im(2/2)</td>
<td>2</td>
<td>2</td>
<td>20</td>
<td>25.3</td>
<td>2 h 5(^*)</td>
</tr>
<tr>
<td>3 B-Im(4/1)</td>
<td>4</td>
<td>1</td>
<td>41</td>
<td>49.8</td>
<td>1 h 5(^*)</td>
</tr>
<tr>
<td>4a B-Im(4/2a)</td>
<td>4</td>
<td>2</td>
<td>29</td>
<td>16.6</td>
<td>2 h 50(^*)</td>
</tr>
<tr>
<td>4b B-Im(4/2b)</td>
<td>4</td>
<td>2</td>
<td>26</td>
<td>5.5</td>
<td>&gt;12 h</td>
</tr>
<tr>
<td>5 B-Im(4/3)</td>
<td>4</td>
<td>3</td>
<td>18/22</td>
<td>~1</td>
<td>—(^*)</td>
</tr>
<tr>
<td>6 B-Im(8/1)</td>
<td>8</td>
<td>1</td>
<td>41</td>
<td>45.1</td>
<td>1 h 15(^*)</td>
</tr>
<tr>
<td>7 B-Im(8/2)</td>
<td>8</td>
<td>2</td>
<td>41</td>
<td>29.7</td>
<td>2 h</td>
</tr>
<tr>
<td>8 B-Im(8/2+1)</td>
<td>8</td>
<td>1</td>
<td>49</td>
<td>31.3</td>
<td>1 h 45(^*)</td>
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<tr>
<td>9 B-Im(16/1)</td>
<td>16</td>
<td>1</td>
<td>70</td>
<td>38.5</td>
<td>2 h 10(^*)</td>
</tr>
<tr>
<td>10 B-Im(8/4+1)</td>
<td>8</td>
<td>4</td>
<td>23/31/41</td>
<td>29.1</td>
<td>2 h</td>
</tr>
<tr>
<td>11 B-Im(12/4+2)</td>
<td>12</td>
<td>4</td>
<td>23/31/51</td>
<td>33.8</td>
<td>1 h 40(^*)</td>
</tr>
<tr>
<td>12 B-Im(24/4+2)</td>
<td>24</td>
<td>2</td>
<td>41/79</td>
<td>49.9</td>
<td>1 h</td>
</tr>
<tr>
<td>13 B-Im(32/4+2)</td>
<td>32</td>
<td>1</td>
<td>41/98</td>
<td>20</td>
<td>2 h 30(^*)</td>
</tr>
</tbody>
</table>

\(^{1}\)The structures of the conjugates are shown in Figure 1 and in Supplementary material (SM-Figure 1).

\(^{2}\)The type of anchor groups of the conjugates as shown in Figure 1(a). Type 1: cyclohexanol moiety; 2: 5\(^*\)-aminimidazole residue; 3: nonnucleotide insertion; 4: 2\(^*\)-modified uridine residue.

\(^{3}\)The number of simple C–C, C–N, or P–O bonds between the 5\(^*\)-terminal phosphate group of oligonucleotide B and imidazole groups of RNA-cleaving construct.

\(^{4}\)Efficiency of the RNA cleavage was measured as percentage of tRNA\textsuperscript{Phe} cleavage at C63-A64 phosphodiester bond achieved after incubation during 1 h in the presence of 10 μM of the conjugate.

\(^{5}\)Time to reach 50% of tRNA cleavage under the standard conditions (see above) at conjugates concentration 10\(^{-5}\) M.

\(^{6}\)50% of tRNA\textsuperscript{Phe} cleavage was not achieved under experimental conditions.

\(^{*}\)These conjugates are described in the preceding paper [1].

The RNA cleaving oligonucleotide conjugates have been prepared in two-step procedure described in [21, 22]. In the first step, the precursor 17-mer GATCGAACACAGGACCT (B) was built up with multiple methoxyxalamido (MOX) modifiers of different type (see Supplementary Material available online at doi:10.4061/2011/748632 for the structures of all conjugates synthesized in this study). In the second step, the prepared oligonucleotide-MOX precursors were functionalized with histamine to yield after deprotection step the oligonucleotide conjugates B-Im(N/m), bearing from 4 to 32 histamine residues at the 5\(^*\)-end.

Figure 1 provides structures of oligonucleotide conjugates B-Im(4/n) and B-Im(8/1) studied in this work. The conjugates hereafter were referred to as B-Im(N/m), where B-Im designates the oligonucleotide-imidazole conjugates, N shows the number of imidazole residues, and m indicates the type of anchor group. In the cases, when two or three conjugates contain equal number of imidazole residues and the same anchor group, the conjugate name is shown in the brackets. The distance between oligonucleotide and imidazole residues of the cleaving part was varied by altering the structures of the anchor and the linker groups.

Cyclohexyl residue was exploited as anchor group in the conjugate Type 1. Thymidine nucleoside was used to anchor the catalytic part in the conjugate Type 2. The thymidine nucleoside, presenting in the structure of conjugate, was supposed to be involved in base pairing with RNA or might be stacked on heteroduplex contributing to the duplex stability. In the conjugate Type 3, an anchor group was chemical construct mimicking phosphodiester bond and used as a rule as nonnucleotide insertion in oligonucleotide. Type 4 anchor group was used in the conjugates 10–13 to increase the number of branches, bearing imidazole residues. For this purpose and to increase the linker length and flexibility, the anchor groups of Types 1 and 2 were incorporated also into the linker structures (see Figure 1-SM, conjugates 6–9 and 11–13). Moreover, the length and structure of linkers, connecting imidazole residues and anchor, were distinguished sufficiently among the compounds (Table 1 and Figure 1-SM). Conjugates containing equal number of imidazole residues, for example, B-Im(4/m) or B-Im(8/m), differ not only in anchor groups, but also in a linker length. The linker of conjugate B-Im(4/3) was the shortest one. In this conjugate, four imidazole residues were placed nonsymmetrically in respect to the phosphate groups forming the anchor, which connected the oligonucleotide B and the cleaving construct.

The identity of the conjugates was confirmed by mass-spectrometry; (see Figure 1). In addition, the purity of the conjugates was confirmed by electrophoresis in 12% PAAM/8 M urea gel (Figure 1(c)). It is seen that the purity of the conjugates is close to 95%. Only in the case of conjugates 2,
3.2. Cleavage of Yeast tRNA\textsuperscript{Phe} by the Conjugates Bearing Multiple Imidazole Residues. Yeast tRNA\textsuperscript{Phe} was chosen deliberately as a target to assess cleavage activity of the conjugates. The rationale behind this was that other oligonucleotide-based aRNases including three prepared using precursor approach had been tested using this target [1, 12, 14–16, 28]. Figure 2(a) represents cloverleaf structure of yeast tRNA\textsuperscript{Phe} showing the target site for the conjugates B-Im(N/m). The recognition element of the studied aRNases was represented by the 17-mer oligonucleotide B, which was complementary to the sequence 44-60 and was designed to deliver the imidazole residues to the sequence 61CACAG65 in the T-arm of the tRNA\textsuperscript{Phe}, which is known to be highly sensitive towards cleavage [29–32]. It was previously shown that oligonucleotide B could efficiently bind to this sequence in the tRNA\textsuperscript{Phe}, which resulted in unfolding of entire t\textsuperscript{3}ΨC hairpin, whereas the aminoacceptor and anticodon stems of the tRNA were only slightly affected by the oligonucleotide binding [33–35].

The conjugates B-Im(N/m) were designed to catalyze RNA cleavage via transesterification reaction. To compare the ribonuclease activities of the conjugates, we used the conditions of single reaction turnover ([B-Im(N/m)] \textgreater [RNA]). The oligonucleotide part of the conjugate provides efficient hybridization with target sequence in tRNA\textsuperscript{Phe} and formation of the stable heteroduplex (K\textsubscript{d} = 7 μM ± 1 μM; V. Petyuk, unpublished data). We believe that under the experimental conditions the rate and efficiency of site-selective cleavage of the target is affected only by the catalytic part of the conjugate.

Site-selective cleavage of [3'\textsuperscript{-32}P]tRNA\textsuperscript{Phe} by the conjugates was carried out at 37°C in 50 mM imidazole buffer. The reaction was initiated by addition of the conjugate B-Im(N/m) to the reaction mixtures. Localization of cleavage sites was performed by comparison of products obtained upon cleavage of tRNA by the conjugates with the products of random RNA cleavage with RNase T1 and 2 M imidazole buffer, pH 7.0 [25, 36]. Figure 2(b) shows typical results of the analysis of tRNA\textsuperscript{Phe} cleavage by the conjugates B-Im(N/m) at 37°C under standard conditions. No measurable spontaneous degradation of tRNA under the experimental conditions in the absence of reagents and in the presence of unmodified parent oligonucleotide B occurred (Figure 2(a), lanes C1 and C2). The conjugates cleave tRNA\textsuperscript{Phe} with high rate mostly at the phosphodiester bonds C63-A64 and A64-G65. The extents of tRNA\textsuperscript{Phe} cleavage at these bonds are about 70% and 20%, respectively. Moreover, some minor cleavage at the phosphodiester bonds C61-A62 and A62-C63 within the target sequence is observed (Figure 2(a), lanes 1–10). The ratio between the products of tRNA cleavage at these phosphodiester bonds located in close proximity to each other in the target sequence seems to correspond to steric accessibility of these bonds to cleavage by the conjugates B-Im(N/m). In the case of conjugate B-Im(4/2a) (similar cleavages were observed for the conjugates with 16, 24, and 32 imidazole residues), slight cleavages are observed at U8-A9 and C75-A76 phosphodiester bonds (less than 3% of the total extent of tRNA cleavage), known as well as C61-A62 and C63-A64 bonds in the yeast tRNA\textsuperscript{Phe} to be very sensitive toward cleavage by various agents [29–32]. The bond U8-A9 is located in the proximity to sequence C61-G65 if tertiary structure of tRNA\textsuperscript{Phe} is taken into account. Thus, one may assume that the observed cleavage of this bond occurs in complementary complex formed by conjugate B-Im(4/2a) with tRNA and is the result of flexibility of RNA-cleaving group of the conjugates. Besides, we can assume that initial cleavage of phosphodiester bond C63-A64 produces a nick in tRNA molecule that may increase the flexibility of the complex RNA-conjugate B-Im(4/2a) and thus makes phosphodiester bonds C64-G65 and U8-A9 available for catalyst’s function groups. The slight cleavage observed at C75-A76 site apparently is the result of spontaneous tRNA hydrolysis in the duplex with the conjugate.

Competition experiments were used to prove that tRNA cleavage occurs in RNA-conjugate complex. tRNA\textsuperscript{Phe} cleavage by conjugate B-Im(4/1) was performed in the presence of oligonucleotide inhibitors of two types: parent oligonucleotide B and oligonucleotide A complementary to the sequence 61-75 (Figure 2(b), lanes 17–19). As expected, the cleavage of tRNA\textsuperscript{Phe} by conjugate B-Im(4/1) is considerably decreased in the presence of oligonucleotides B (primary data not shown) and is completely abolished in the presence of oligonucleotide A, which protects target sequence by duplex formation. It is worth noting that in the presence of oligonucleotides B, the cleavage of tRNA\textsuperscript{Phe} is suppressed in all sites due to competitive binding, whereas oligonucleotides A inhibit tRNA\textsuperscript{Phe} cleavage only at the target sequence failing to change reaction rate at other sites. These results indicate the structural specificity of cleavage and stress the fact that the cleavage occurs only at a single-stranded target sequence.

Figure 2(c) shows concentration dependencies of the cleavage reaction for conjugates B-Im(4/1) (3) and Im(24/4+2) (11) (curves 1 and 2, resp.) and binding of the conjugate B-Im(4/2a) and parent oligonucleotide B with tRNA (curves 3 and 4, resp.). It is seen that the cleavage data are in agreement with the complex formation. Furthermore, the binding affinity of the parent oligonucleotide and the conjugate studied under identical conditions are found to be similar (Figure 2(c)). Therefore, the conjugation of bulky imidazole-containing reactive groups to the oligonucleotide does not affect the hybridization process.

To estimate the influence of buffer on efficiency of site-selective RNA cleavage, we compared tRNA\textsuperscript{Phe} cleavage by the conjugate B-Im(4/2) in different buffer solutions (Figure 2(d)), taken 50 mM imidazole buffer, pH 7.0 as a standard conditions. The rate of site-selective cleavage is affected by replacement of 50 mM imidazole buffer, pH 7.0, by 50 mM Tris-HCl buffer or 50 mM cacodylate buffer at pH 7.0 by the factor of 1.5 indicating that imidazole itself may contribute to cleavage reaction. At longer incubation time, this difference became insignificant and does not exceed estimated experimental error. Surprisingly, 50 mM HEPES buffer entirely abolishes cleavage inactivating the conjugates. The reason of this is not entirely understood.
Figure 2: Site-selective cleavage of yeast tRNA\textsuperscript{Phe} by the oligonucleotide conjugates bearing multiple imidazole residues in the catalytic part.
One explanation could be that sulfonic acid of HEPES [4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid] may inter-act with protonated imidazole residues of the conjugates and inactivate it. Previously, we also observed twofold decrease of the rate of RNA cleavage by oligonucleotide conjugate bearing two imidazole residues in 50 mM HEPES-KOH buffer, whereas the other buffers only slightly affected the rate of RNA cleavage [37].

In Figure 2, (a) represents cloverleaf structure of yeast tRNA_{Phe} and target sequences for oligonucleotide B-based artificial ribonucleases B-Im(N/m). Bold letters show target sequence for oligonucleotide-competitor A. Arrows indicate the sites of site-selective tRNA cleavage by the conjugates; (b) represents cleavage of tRNA_{Phe} with oligonucleotide conjugates B-Im(4/m). Autoradiograph of 12% polyacrylamide 8/8 M urea gel. Cleavage reactions were performed at 37°C in 50 mM imidazole buffer, pH 7.0, containing 200 mM KCl, 0.2 mM EDTA, 100 μg/mL RNA carrier, 5 × 10^{-7} M [3′-32P]tRNA_{Phe} and 1 × 10^{-5} M one of the conjugates B-Im(4/1), B-Im(4/2a), and B-Im(4/2b). Lanes L and T are Imidazole and RNase T1 ladders, respectively. Lanes C1 and C2 are tRNA incubated in the reaction buffer for 8 h in the absence and in the presence of oligonucleotide B (5 × 10^{-5} M), respectively. tRNA cleavage by conjugate B-Im(4/1) (3) for 0.5, 1, 3, 5, and 8 h (lanes 1–5, resp.), by conjugate B-Im(4/2a) (4a) for 0.5, 1, 3, 5, and 8 h (lanes 6–10), by the conjugate B-Im(4/2b) (4b) for 0.5, 1, 3, 7, 10, and 18 h (lanes 11–16); by the conjugate B-Im(4/1) (3) in the presence of oligonucleotide A (1 × 10^{-4}) for 0.5, 1, and 5 h (lanes 17–19). Concentration of the conjugates in the reaction mixtures was 1 × 10^{-5} M. Reactions were quenched and analyzed as described in the experimental part; (c) represents concentration dependencies of the tRNA_{Phe} cleavage by the conjugates B-Im(4/1) (3) and B-Im(24/2) (12) (curves 1 and 2, resp.) and complex formation with the conjugate B-Im(4/2a) (4a) (curve 3) and parent oligonucleotide B (curve 4). The reaction mixtures were incubated for 2 h under the conditions described above; (d) represents the influence of the buffer nature on the rate of tRNA_{Phe} cleavage by the conjugate B-Im(4/2a) (4a). [3′-32P]tRNA_{Phe} (0.5 μM) was incubated with 2 μM of (4a), in 50 mM imidazole buffer, pH 7.0 (Im) or 50 mM cacodylate buffer, pH 7.0 (Cac) or 50 mM Tris-HCl buffer, pH 7.0 (Tris) or 50 mM HEPES buffer, pH 7.0 (Hepes) containing 0.2 M KCl, 0.2 mM EDTA, and 0.1 mg/mL RNA carrier.

3.3. Kinetics of tRNA_{Phe} Cleavage by the Conjugates. Figure 3(a) displays the kinetics of tRNA_{Phe} cleavage with conjugates under the study taken at conjugate concentration 10 μM. It is seen that the conjugates cleave RNA with different efficacy. One of the most effective conjugate is B-Im(4/1), as complete site-selective cleavage of the target by this conjugate is achieved within 2-3 hours. Similar kinetics are displayed by the conjugate B-Im(8/1) (6) and the B-Im(24/4+1-2) (12). The conjugates B-Im(4/2a) (4a) and B-Im(8/2) (7) catalyze RNA cleavage less efficiently as compared with B-Im(4/1) or B-Im(8/1). Cleavage efficiency of the conjugate B-Im(4/3) (5) was essentially poor.

Figure 3(b) shows kinetic curves of tRNA_{Phe} cleavage by conjugate B-Im(4/1) obtained at different concentrations of the conjugate. The character of kinetic curves evidences the bimolecular reaction: the rate of RNA cleavage is increased with the increasing of the conjugate concentrations. At concentration of conjugate B-Im(4/1) of 10 μM, which provides its almost quantitative binding to tRNA, the half-life of tRNA is about 1 h (Figure 3(b), curve “10 μM”). When concentration of conjugate B-Im(4/1) is increased up to 50 μM, the half-life of tRNA is counted by minutes (Figure 3(b), curve “50 μM”). It should be noted that the site-selective cleavage of tRNA proceeds much slower than the binding of the parent oligonucleotide B to tRNA under similar conditions [33–35]. At concentration of the conjugate equal to or twice as higher as concentration of the tRNA, the curves reveal lag period (Figure 3(b), curves “0.5” and “1 μM,” resp.) indicating that the process of site-selective RNA cleavage includes several consecutive steps occurring with comparable rates. Another explanation of this could be that the reaction has more complex character and goes through some stages other than binding of the conjugate and cleavage, which results in sigmoid-like kinetic curve.

For conjugate B-Im(4/1), we calculated the association rate and cleavage rate constants from the data shown in Figure 3(b), assuming that cleavage of the tRNA occurs within the complex with the conjugate. In this case, hybridization step is the second-order reaction and cleavage stage is intramolecular first-order reaction. It turns out that cleavage pe se occurs 50 times faster than the binding of the conjugate with the complementary sequence within RNA target (association rate constant and cleavage rate constant are (0.074 ± 0.002) M^{-1} s^{-1} and (3.4 ± 0.55) × 10^{-3} s^{-1}, resp.). In other word, binding of the conjugate with tRNA is the rate-limiting stage of site-selective RNA cleavage.

3.4. Correlations between Structure and Activity of the Conjugates Bearing Multiple Imidazole Groups. The conjugates display different cleavage activities (Table 1) ranging from very high cleavage rate (τ_{1/2} ~ 1 h in the case of B-Im(4/1)) to very low (τ_{1/2} value was not achieved under the experimental conditions in the case of B-Im(4/3)). Remarkable cleaving activity of the conjugates 3, 6, and 12 as well as moderate cleavage activity of the conjugates 4a, 8, 10, 11, and 13 and poor efficiency of the conjugate 5 are likely resulted from the differences in the structure of the catalytic part of the conjugates rather than due to different hybridization properties and the cleavage reaction pe se. The conjugates under the study contain identical oligonucleotide recognition element and display similar cleavage specificity with only minor differences in the cleavage pattern. Therefore, neither hybridization nor sensitivity of the phosphodiester bonds toward the cleavage could explain the differences in the catalytic properties of the conjugates. Even the catalytic parts of the conjugates in several cases contain the equal number of imidazole residues.

Table 1 summarizes data on the cleavage activity and structure of the conjugates under the study (linker length in
total number of simple C–C, C–N, C–O, and P–O bonds between 5′-end of oligonucleotide B and imidazole residues, type of anchor group, and reaction half-times). It is seen that hydrolytic activity of compounds is in a good agreement with the length of their linkers (Table 1). Amongst the conjugates bearing four imidazole residues, the highest cleavage activity is observed for the conjugate B-Im(4/1), which has the linkers of 41 simple bonds. The conjugates B-Im(4/2a) with the linkers of 29 chemical bonds exhibit 3-fold lower cleavage activity, whereas B-Im(4/3) (18/24 bonds) cleaves tRNA with lowest efficiency. Thus, stepwise shortening of a linker part of conjugate B-Im(4/m) from 41 to 18/24 simple bonds results in decreasing of cleavage efficiency from 50% per hour to 1% per hour. As it is seen from Table 1, only the conjugates containing the linkers of 40 simple bonds are capable of efficiently catalyzing cleavage of tRNA\textsuperscript{Phe} in a site-selective manner. Thus, we can conclude that a long flexible linker of 40 or more simple C–C, C–N, or P–O bonds is required for efficient site-selective cleavage of RNA target.

Figure 4 display Structure-Activity correlations found for the conjugates B-Im(N/m). It is seen that cleavage activity of the conjugates B-Im(N/1) depends on the number of imidazole groups in the catalytic part (Figure 4(a)). The conjugates bearing 4 and 8 imidazole residues exhibit the highest cleavage activity, while increasing of the number of imidazole groups inhibits cleavage. It is worth pointing out that sigmoidicity of kinetic curves taken under identical conditions is increased with increasing of the number of imidazoles in the conjugate (primary data not shown). It is likely that cleavage activity of these conjugates is affected by a steric interference between imidazoles, thus leading to a loss of cleavage rate. Therefore, four (and/or eight) imidazole residues in the catalytic part of the conjugate seem to be sufficient for efficient RNA cleavage.

From the data shown in Figure 4(b), it is seen that ribonuclease activity of the conjugates depends on type of the anchor group used for attachment of parent oligonucleotide to RNA-cleaving construct. The conjugates with Type 1 anchor group (B-Im(N/1)), cyclohexyl moiety (Figure 1(a)), display higher cleavage activity than conjugates with the anchor group in a form of thymidine residue (Type 2, Figure 1) and especially than conjugates with a nonnucleoside anchor group (Type 3, Figure 1) (Table 1). The difference between cleavage activity of the conjugates with Type 2 and Type 4 anchor groups does not exceed experimental error, but is reliably lower than that for conjugates with Type 1 anchor group. Thus, the structure and flexibility of the linker part, the number of imidazole residues in the catalytic part, and the type of anchor group affect cleavage activity of the conjugates and play a role in the optimal positioning of the catalyst in respect to scissile phosphodiester bond.

3.5. Molecular Modeling Study. The main goal of the molecular modelling was to clarify the following issues.

1. Is there any preferable orientation(s) of the cleaving group(s) in the vicinity of the cleavage site, which could explain the hydrolytic activity (or its absence) of each conjugate?

2. Is there any evidence of interaction(s) between the cleaving constructions and the DNA:RNA hybrid, which could stabilize preferable orientation(s) of these groups near the cleavage site?

3. What is the correlation between the structure of the cleaving constructs and the linker groups and their hydrolytic activity?

It is evident from the biochemical assays that hydrolytic activity within the heteroduplex tRNA\textsuperscript{Phe}-conjugate is governed by the intrinsic properties of the cleaving constructs, but not the detailed structure of the DNA-RNA hybrid, which was the same in all experiments. Therefore, the main priority was to identify possible location(s) of the cleaving

![Figure 3: Kinetics of tRNA\textsuperscript{Phe} cleavage by the conjugates: (a) tRNA cleavage by B-Im(4/1) (3) (rhombs), B-Im(4/2a) (4a) (squares), B-Im(4/3) (5), (asterisks), B-Im(8/4+1) (10) (triangles), and B-Im(24/4+2) (12) (crosses) under standard conditions at conjugates concentration 10 \( \mu \text{M} \). (b) tRNA cleavage by B-Im(4/1) (3) at concentrations ranging from 0.5 to 50 \( \mu \text{M} \).]
group(s) relatively to the cleavage site rather than to determine the detailed conformation of the hybrid (which would be impossible in the absence of relevant NMR/X-ray data).

It has been shown earlier that hybridization of long oligonucleotides to TΨC-loop [33, 38] or to the 3′-acceptor stem [33–35] of tRNA induced a local unfolding of the tRNA secondary structure. Therefore, we assumed that the hybrid part of the molecules under the study can be represented to a first approximation by a double-stranded DNA:RNA duplex, which would form primarily an A-like conformation, according to physicochemical investigations [39–41]. In our molecular modeling studies, we used a shortened model for the heteroduplex, comprising the target RNA sequence (corresponding to the 5′A44–U69 fragment of tRNA^Phe^) and the oligodeoxyribonucleotide conjugate complementary to this RNA region (Figure 2(a)).

Based on this hybrid duplex, three model molecules were created (M-Im(4/1), M-Im(4/2a), and M-Im(4/2b)) by attachment of the respective cleaving constructs to the 5′ terminal phosphate group of the G1 nucleotide residue (see Figures 1 and 2). At this stage, in the absence of the detailed structural data for the hybridized heteroduplex, it was reasonable to treat the hybrid as a “solid” structure [24] whilst allowing cleaving constructs to possess a full conformational flexibility during the calculations.

For each molecule M-Im(4/1), M-Im(4/2a), and M-Im(4/2b), several starting structures were created differing in the spatial orientation of the cleaving construct(s), ranging from “in” to “out” extremes (see “Section 2”). This increased the conformational space searched for each model molecule. All starting structures were subjected to independent simulating annealing (see “Section 2”) giving rise to the respective final structures (M-Im(4/1): F1-Im(4/1)–F9-Im(4/1); M-Im(4/2a): F1-Im(4/2a)–F9-Im(4/2a); M-Im(4/2b): F1-Im(4/2b)–F9-Im(4/2b)).

Both structural parameters and energy values of each final structure were analyzed and compared with those of “active” conformations (O1-Im(4/1), O2-Im(4/1), O1-Im(4/2a), O2-Im(4/2a), O1-Im(4/2b), and O2-Im(4/2b)), which had deliberately preorganized cleaving centers according to [20, 26, 27, 42, 43] (see “Section 2”) (Table 2).

Molecules containing four imidazole residues M-Im(4/1), M-Im(4/2a), and M-Im(4/2b), having two bis-imidazole cleaving constructs, differ in the geometrical properties of their linker groups (see Figure 1). The linker group of M-Im(4/1) possess a cyclohexyl fragment connecting the cleaving moieties with the oligonucleotide. In M-Im(4/2a), the cyclohexyl fragment is replaced by a deoxyribothymidine fragment. M-Im(4/2b) is a shorter analogue of M-Im(4/2a).

For each molecule, nine final structures plus two “optimal” conformations were obtained as a result of the simulated annealing calculations. The data on the distances between N1 atoms of imidazole rings and target site as well as energy values are presented in Table 2.

For M-Im(4/1) (Table 2), the most energetically favorable conformations (O1-Im(4/1), O2-Im(4/1), and F4-Im(4/1)) are characterized by the location of at least two imidazole groups close to the target site (3–7 Å). In the most cases, M-Im(4/2a) conformers have at least one imidazole group located near the cleavage site, which suggests a high probability for cleaving groups to form a preorganized
Table 2: Distances between active centers of the conjugates B-Im(4/4) and RNA target atoms.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Final structure</th>
<th>Energy (kcal/mol)</th>
<th>ΔE (kcal/mol)</th>
<th>Dist. Pair 1(E)</th>
<th>Dist. Pair 2(E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-Im(4/1)</td>
<td>F1- Im(4/1)</td>
<td>47.3</td>
<td>18.2</td>
<td>13</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>F2- Im(4/1)</td>
<td>66.7</td>
<td>37.6</td>
<td>32.4</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>F3- Im(4/1)</td>
<td>48.3</td>
<td>19.2</td>
<td>14.0</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>F4- Im(4/1)</td>
<td>28.6</td>
<td>-0.6</td>
<td>-5.8</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>F5- Im(4/1)</td>
<td>60.2</td>
<td>31.1</td>
<td>25.9</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>F6- Im(4/1)</td>
<td>42.6</td>
<td>13.5</td>
<td>8.3</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>F7- Im(4/1)</td>
<td>54.9</td>
<td>25.8</td>
<td>20.5</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>F8- Im(4/1)</td>
<td>47.3</td>
<td>18.2</td>
<td>13.0</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>F9- Im(4/1)</td>
<td>49.8</td>
<td>20.7</td>
<td>15.5</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>O1- Im(4/1)</td>
<td>-2.4</td>
<td>—</td>
<td>—</td>
<td>3.17</td>
</tr>
<tr>
<td></td>
<td>O2- Im(4/1)</td>
<td>-0.9</td>
<td>—</td>
<td>—</td>
<td>17.7</td>
</tr>
</tbody>
</table>

| M-Im(4/2a) | F1- Im(4/2a)     | -6.7             | -4.3         | -5.8           | 12.4           | 25.1           | 26.4           | 19.8           |
|            | F2- Im(4/2a)     | 24.0             | 26.3         | 24.8           | 23.1           | 15.6           | 23.2           | 34.8           |
|            | F3- Im(4/2a)     | 0.5              | 2.9          | 1.4            | 21.1           | 11.6           | 21.3           | 19.8           |
|            | F4- Im(4/2a)     | 9.7              | 12.0         | 10.5           | 6.4            | 15.8           | 23.3           | 27.8           |
|            | F5- Im(4/2a)     | 1.7              | 4.0          | 2.5            | 5.6            | 10.1           | 21.6           | 26.7           |
|            | F6- Im(4/2a)     | 23.4             | 25.8         | 24.3           | 26.1           | 30.6           | 16.2           | 8.6            |
|            | F7- Im(4/2a)     | 23.4             | 25.8         | 24.3           | 26.1           | 30.6           | 16.2           | 8.6            |
|            | F8- Im(4/2a)     | 0.5              | 2.9          | 1.4            | 21.2           | 11.6           | 24.4           | 17.4           |
|            | F9- Im(4/2a)     | 13.3             | 15.6         | 14.1           | 14.4           | 18.4           | 28.2           | 27.8           |
|            | O1- Im(4/2a)     | -2.4             | —            | —              | 3.17           | 3.0            | 20.1           | 33.7           |
|            | O2- Im(4/2a)     | -0.9             | —            | —              | 17.7           | 17.6           | 3.0            | 3.2            |

| M-Im(4/2b) | F1- Im(4/2b)     | -4.7             | 6.9          | -16.2          | 23.7           | 15.9           | 21.4           | 19.9           |
|            | F2- Im(4/2b)     | -17.9            | -6.3         | -29.3          | 30.9           | 32.7           | 21.9           | 30.7           |
|            | F3- Im(4/2b)     | -17.8            | -6.2         | -29.2          | 27.6           | 36.0           | 25.9           | 21.9           |
|            | F4- Im(4/2b)     | 0.1              | 11.7         | -11.3          | 5.3            | 23.3           | 14.5           | 14.3           |
|            | F5- Im(4/2b)     | -9.1             | 2.5          | -20.5          | 21.1           | 26.2           | 23.5           | 25.8           |
|            | F6- Im(4/2b)     | 0.1              | 11.7         | -11.3          | 18.5           | 9.2            | 11.3           | 19.7           |
|            | F7- Im(4/2b)     | 0.1              | 11.7         | -11.3          | 18.5           | 9.2            | 11.3           | 19.7           |
|            | F8- Im(4/2b)     | -6.7             | 4.9          | -18.1          | 9.0            | 10.6           | 20.7           | 23.3           |
|            | F9- Im(4/2b)     | -2.5             | 9.1          | -13.9          | 14.9           | 30.4           | 27.9           | 31.8           |
|            | O1-Type3         | -11.6            | —            | —              | 3.1            | 3.0            | 13.4           | 14.9           |
|            | O2-Type3         | 11.4             | —            | —              | 25.2           | 27.2           | 3.0            | 3.0            |

(a) Im1-OH represents the distance between N1 of one of the imidazole residues and the 2′OH group of C63.
(b) Im2-OP represents the distance between N1 of the other imidazole residue and the oxygen of the phosphate group connecting C63 and A64.

“active” conformation. These data are in agreement with the biochemical assays on high hydrolytic activity seen for B-Im(4/4) conjugate, showing that this compound reaches the maximum activity (49.8%) found for artificial ribonucleases. Figure 5 represents the fragments of the F4-Im(4/1) structure, as the most energetically favorable conformation of M-Im(4/1), and shows the probable orientation of the cleaving groups.

In the case of M-Im(4/2a), most of the final structures obtained in the simulated annealing experiments are characterized by a distant location of cleaving groups from the target site (Table 2). For example, the lowest-energy structures F1-Im(4/2a), F3-Im(4/2a), and F8-Im(4/2a) have very unfavorable orientations of imidazole cleaving groups in terms of hydrolytic activity (see Figure 6 showing F1-Im(4/2a) final structure as an example). The possible reason of this could be the presence of a deoxyribothymidine fragment linking the oligonucleotide and the cleaving construct. According to our data, the thymidine base is involved in the DNA:RNA helix structure in a semistacking interaction with the neighboring nucleotide residue dG1. The thymidine base matches comfortably the duplex structure, although no additional hydrogen bonding with the opposite strand was detected (for instance, with the A62). This interaction could control the overall conformation of the rest of the cleaving construct and possibly restrict the conformational freedom of this group, resulting in a decreased probability to render an “active” conformation. This could explain the relatively
low hydrolytic activity of B-Im(4/2a) (16.6%) compared to B-Im(4/1).

Even more pronounced decrease in hydrolytic activity (down to 5–7%) was observed for conjugate B-Im(4/2b), a structural analogue of B-Im(4/2a) with a shortened linker group. Data obtained in biochemical assays are consistent with results from molecular modeling for M-Im(4/2b) (Table 2), showing that the most energetically favorable conformations (for instance, F2-Im(4/2b), F3-Im(4/2b), and F5-Im(4/2b)) have a distant location of the cleaving groups relative to the target site (Figure 7 shows F5-Im(4/2b)). Moreover, only one of two bis-imidazole groups can occupy a position, favorable for cleaving activity. This decreases the statistical probability of reaching an “active” conformation for M-Im(4/2b). Generally, most of the final structures of M-Im(4/2b) are characterized by long distances between imidazole rings and target atoms. Analysis of the structural parameters showed that the length of the linker group is not enough to provide a good chance to reach the target site. This possibly contributes to the restricted conformational freedom caused by thymidine interactions with the RNA:DNA duplex structure (see above for M-Im(4/2a)). Taken together, these explain the lowest hydrolytic activity for B-Im(4/2b) between the artificial ribonucleases.

Analysis of final structures resulting from the simulating annealing experiments shows a remarkable ability of all cleaving constructs to form multiple hydrogen bonds that generally can be represented by two types (Scheme 1). The first type is the intermolecular interactions between cleaving constructs and the RNA target, which are formed by oxygen atoms of the RNA phosphate backbone and either (i) by imidazole rings of the cleaving groups or (ii) by protons of the linker amido groups (see Scheme 1). These types of hydrogen bonding seem to be in favor of the hydrolytic activity of compounds due to their ability to stabilize the location of cleaving groups, close to the RNA target. The second type of possible hydrogen bonding (Scheme 1) refers to intramolecular interactions within cleaving constructs themselves and involves interactions between imidazole rings and amido groups, carbonyl groups and phosphate groups of the linker. This type of hydrogen bond interaction also includes intralinked bridges formed by amido and carbonyl/phosphate groups. Intramolecular hydrogen bonds seem to compete with favorable intermolecular interactions, preventing the imidazole groups from appropriate contacts with
Based on these results, we propose several suggestions on how to improve further the cleaving potential of artificial ribonucleases. First, it is important to avoid the use of any carbonyl/phosphate groups within the linker in order to decrease the possibility of intramolecular hydrogen bonding. Secondly, it could be advantageous to introduce polycationic groups within the linker, which might increase possible contacts of cleaving groups with negatively charged phosphate backbones of target RNA. Finally, the insertion of a suitable hydrophobic/intercalating groups within the linker might also increase chances for these groups to be located in the vicinity of the RNA target due to additional stacking/hydrophobic interactions with different ribonucleotide environments.

4. Conclusions

In the current research, we studied oligonucleotide-based artificial ribonucleases containing multiple imidazole residues in the catalytic part of the aRNases with systematically varied structure of cleaving constructs. All the conjugates contain the same addressing oligonucleotide which provides efficient and almost quantitative binding of the conjugates to the target sequence within the tRNA. This allows comparing different cleaving constructs in terms of site specificity and efficiency of RNA cleavage. Obtained results let us conclude that the efficiency of site-selective RNA cleavage is governed by a number of dynamic parameters among which the most important is the flexibility of a linker between the catalytic imidazole residues and the addressing oligonucleotide. The other factors affecting the efficiency of site-selective RNA cleavage are the number of imidazole residues in the catalytic part, the type of anchor group, connecting linker structure and the oligonucleotide, and the length of a linker between the catalytic imidazole groups of the construct and the oligonucleotide. We found that four imidazole residues located in the catalytic part of the conjugate can efficiently catalyse cleavage of phosphodiester bond in the case when they are located in the close proximity of RNA sugar-phosphate backbone, while increasing of the number of imidazole groups inhibits cleavage, possibly due to steric interference between the imidazole residues.

Molecular modeling carried out to explain differences in ribonuclease activity of the conjugates showed that preferable orientation(s) of cleaving constructs strongly depend on the structure of the anchor group and length of the linker. The inclusion of deoxyribothymidine as an anchor group significantly reduced the probability of cleaving groups to locate near the cleavage site due to a stacking interaction with the neighbouring nucleotide residue. Remarkably high cleavage activity was displayed by the conjugates with the most flexible and extended cleaving construct, which presumably provides a better opportunity for imidazole residues to be correctly positioned in the vicinity of scissile phosphodiester bond.

The other very important factor affecting the efficiency of site-selective RNA cleavage is sensitivity of phosphodiester bonds of RNA toward cleavage. Different catalytic structures display some sequence preference [1]. On the other hand, phosphodiester bonds in different RNA sequence display different sensitivities to cleaving agents [3, 44–48]. These factors should be taken into account when selecting target
sites within specific RNAs. In our experiments, the cleaving groups were targeted to CACA sequence of tRNA\textsubscript{Phe}, known to be of particular sensitivity to cleavage by different agents. So, the ribonuclease activity of the conjugates was compared under favourable conditions. On the other hand, binding of oligonucleotide conjugates to target RNA is followed by RNA structure rearrangements that can bring the target site in favorable or unfavorable positions toward the catalytic groups [32, 49, 50]. Thus, optimisation of the structure of catalytic groups of oligonucleotide conjugates and identification of optimal structures of RNA for targeting with artificial ribonucleases may facilitate the creation of highly specific artificial ribonucleases of a new generation.

Results of the studies performed by the authors revealed the problems to be solved in order to develop the highly efficient RNA cleaving oligonucleotide conjugates. A few important factors determining efficacy of RNA cleavage remain to be investigated so that the oligonucleotide conjugates take advantage of the known mechanisms used by natural enzymes for achieving high reaction rate.

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References


A Potential of microRNAs for High-Content Screening

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1. Introduction

miRNAs (microRNAs) are 17-nt to 24-nt long noncoding RNAs that regulate gene expression in metazoans. miRNAs act by partially or completely complementary binding to their target mRNAs, resulting in translational repression and/or mRNA degradation [1, 2]. miRNAs are predicted to affect the expression of nearly 60% of protein-coding mammalian genes [3, 4] and, thereby, to control many, if not all, biological processes. Fundamental changes at the cellular and organismal level, including development [5], aging [6], the stress response [7], cell proliferation [8, 9], and apoptosis [10, 11], were shown to be regulated by miRNAs. Furthermore, miRNAs have been implicated in various diseases, such as diabetes [12–14], cancer [15, 16], hepatitis C [17], neurodevelopmental (reviewed in [18]), and mental [19] disorders. Rapidly growing knowledge of miRNAs as potent regulators in health and disease makes miRNAs attractive as targets for therapeutic intervention [20, 21] as well as for diagnostic markers [22, 23].

Numerous previous publications have addressed miRNA biogenesis and action (for detailed reviews see [24, 25]). Briefly, miRNAs are transcribed as long primary transcripts (pri-miRNAs), most of which are polyadenylated and capped. Pri-miRNAs are initially cleaved in nucleus by a multiprotein complex, called Microprocessor, yielding ~70-nt long stem-loop structured precursor miRNAs (pre-miRNAs). The key components of the Microprocessor complex are the RNase III enzyme Drosha and the double-stranded RNA-binding protein DGC8/Pasha [26]. The excised pre-miRNA hairpin is then exported to the cytoplasm by Exportin-5 complexed with Ran-GTPase [27]. In the cytoplasm, the pre-miRNA is further processed to a 20–22-nt long miRNA/miRNA* duplex by a second RNase III enzyme, Dicer, which is in a complex with the TRBP and PACT proteins [28, 29]. Subsequently, the miRNA duplex is unwound by multiple helicases, which may be miRNA-specific and may regulate miRNA activity [30, 31]. The miRNA-targeting miRNA strand (guide strand) is loaded into the miRNA-induced silencing complex (miRISC). Until recently, it was assumed that the complementary miRNA* strand (passenger strand) is degraded, but there is now evidence that a substantial cohort of miRNA* species is functionally active [32]. The core components of miRISC are proteins of the Argonaute (AGO) [33] and GW182 protein families [34]. Individual miRNAs might need specific maturation steps [35–37]. Once incorporated into miRISC, the miRNA brings the complex to its target mRNAs by interacting with complementary binding sites, which can be present in multiple
copies [38–40]. Each miRNA can usually affect more than one transcript and, as a consequence, many proteins simultaneously [41, 42]. On the other hand, multiple miRNAs can repress expression of a single target mRNA [43–46]. miRNAs are postulated to preferentially bind to the 3′ untranslated regions (3′UTRs) of transcripts [47]. However, recent experimental evidences show the existence of a new class of miRNA targets containing miRNA binding sites in both their 5′UTR and 3′UTR [48] or within the coding region of mRNA [49]. The complexity of miRNA-mediated modulation of gene expression is only beginning to be appreciated, and much research needs to be done in order to understand miRNA global and adaptive regulatory functions. In this review, we summarize available methodologies for modulating expression levels of endogenous miRNAs, as well as on the application of these strategies for high-content and high-throughput functional studies.

2. RNA Silencing

The discovery that small ncRNAs (noncoding RNAs) play pivotal roles in fundamental biological processes has considerably widened our knowledge of mechanisms of gene regulation in the last years [50, 51]. siRNAs (small interfering RNAs) and miRNAs are the two best characterized classes of ncRNAs. Both are derived from dsRNA (double-stranded RNA) precursors and exert their inhibitory function on gene expression by Watson-Crick base-pairing to complementary sequences in target RNA molecules: an effect commonly referred to as gene silencing [2]. Moreover, both siRNAs and miRNAs share some components of the cellular effector machinery involved in gene silencing [52]. Usually, miRNAs modify expression of endogenous genes whereas siRNAs have evolved to defend genome integrity against foreign invaders, like viruses or transposons [53]. In mammals siRNAs are 21–22 nt long fragments often, but not always, derived from foreign dsRNA by Dicer [52, 54]. siRNAs are incorporated into the siRNA-induced silencing complex (siRISC) [55] and bind to perfectly matched sequences in target molecules. Typically, this induces degradation of the bound RNA, a function called RNAi (RNA interference) [53], and this property was widely implicated in functional studies over the last decade (see what follows).

Binding of miRNA to perfectly matched sequences in mRNA can also result in degradation of the mRNA [56], but usually, miRNAs bind to sites in mRNAs with only partial sequence complementarity. This results primarily in translational repression rather than degradation [57] but can also cause secondary nucleolytic degradation of the mRNA [24]. Conversely, binding of siRNAs to partly unmatched sequences in mRNAs can result in translational blockade-potentially interfering with the results from siRNA-based screening experiments [56].

3. siRNA/shRNA-Based Screens

In the last years RNAi has developed into a powerful tool for systematic studies of fundamental physiological and pathological processes. A number of large-scale screens have been completed, analysing diverse cellular processes. Recent impressive examples of genome-wide RNAi-based screens in human cells are provided by the work of Collinet et al. [58], who performed a high-content survey of genes involved in endocytosis, and by the study of Neumann et al., who used time-lapse microscopy in living cells to identify genes that play a role in cell division [59].

siRNA-based screens have also been performed in several model organisms. C. elegans and Drosophila are especially receptive to this type of genetic screening [60]. In C. elegans, for example, gene silencing can be accomplished by feeding bacteria that express long dsRNA (about 200–2000-bp-long) or by providing such dsRNA in the medium [61, 62]. RNAi in cultured cells of Drosophila can similarly be induced by adding in vitro transcribed dsRNA to the culture medium [63]. In mammalian cells, in contrast, short RNA duplexes of 21–29 bp have to be administered, because long dsRNA evokes a response of the innate immune system, which ultimately leads to apoptosis [64]. The short siRNAs can be chemically synthesized or derived from transcribed PCR products by digestion with recombinant Dicer or bacterial RNAse III (esiRNAs-endoribonuclease-prepared short interfering RNAs) [64, 65]. siRNA-mediated knock-down can attain close to 100% reduction in the target mRNA. However, the effectiveness of an individual siRNA is hard to predict and, therefore, several siRNAs targeting different regions of the target mRNA have to be tested. Some authors suggest checking 4–6 siRNAs per gene to obtain reliable results in a screening experiment [66].

If stable knock-down is needed or if cells are difficult to transfect, short hairpin RNAs (shRNAs) can be used. The sequences encoding the shRNA can be cloned into plasmids or virus-derived vectors (lenti-, retro-, or adenoviral origin) [67–69]. In the case of “second generation” shRNAs, so called shRNA-miRs, the RNAi-triggering small RNA sequence is cloned into the backbone of a pri-miRNA [70]. This design principle, together with improved selection of the small RNA targeting sequence, improves both the production levels of small RNAs and the silencing efficiency. shRNA-miRs can be cloned into constructs carrying different promoters and used for tissue-specific or inducible expression [70].

4. Modulation of miRNA Function as an Approach for Functional Screening

Clearly, their biological importance per se makes analyses of miRNA expression and identification of their target mRNAs a focus of the current research. Yet specific features of miRNAs give them strong potential as tools in functional genomics. Firstly, miRNAs are able to change the translation of hundreds of mRNAs simultaneously and, by doing so, add another layer of regulation to gene expression [71]. In fact, miRNAs can influence the whole biological programs, including development, apoptosis, proliferation, and differentiation, not only through direct interactions with target mRNAs but also indirectly by altering expression of, for example, components of the translation or RNAi machinery [42].
In general, miRNAs effect only subtle modulation of target gene expression. Their pronounced effect on cellular behaviour might, therefore, be a consequence of their ability to influence multiple genes involved in a single pathway. Indeed, miRNA-based screens can provide lists enriched in functionally related targets: for example, a recent RNAi screen identified three target genes that phosphoryc miR-19 and cooperate in the regulation of phosphatidylinositol-3 kinase-mediated survival signaling [72]. miRNA-based screens and, especially, a combination of miRNA- and siRNA-based screens, though laborious, might have a clear advantage over siRNA-based screens alone, since with siRNAs, downregulation of only single individual miRNAs is expected. On the other hand, enrichment of target genes with specific functions is not always found using current bioinformatic prediction tools [73]; therefore, the potential to organise hits of miRNA-based screens directly into functional networks is as yet unproven. The fact that a single mRNA can be inhibited by different miRNAs [74] also means that cellular phenotypes result from synergistic effects, complicating interpretation. Having an infrastructure for large-scale experimentation, complex and previously unanticipated regulatory patterns might be identified.

In contrast to the vast number of mRNAs that have to be knocked-down in comprehensive functional genomic approaches using siRNAs/shRNAs, the number of miRNAs that have to be analysed in a genome-wide screen is relatively low. To date, 1424 miRNAs have been identified in the human genome (mirBase database, release 17; http://www.mirbase.org/). Various in silico methods, developed to predict targets of miRNAs, estimate that between 10% and 60% of all mRNAs might be influenced by miRNAs [3, 4, 75, 76]. Hence, the analysis of the comparatively small number of miRNAs could, in principle, cover the function of substantial fraction of human genes. To modulate the expression of the same number of genes by siRNA/shRNA would require about 44,000 to 66,000 different siRNA/shRNAs, taking into account the need for multiple reagents targeting the same mRNA (see above).

5. Tools to Modulate miRNA Function

Here, we will briefly describe approaches that are currently in use to modulate miRNA function and discuss their potential and limitations (Table 1). Basically, it is possible either to interfere with miRNA expression (loss-of-function assays) or to induce ectopic (over-) expression of miRNA (gain-of-function).

One possibility for analysis of miRNA would be a complete knock-out. This very precise intervention will result in a complete loss of function. The resulting phenotypes are, therefore, often stronger than those seen after knock-down. For some time, generation of knock-out phenotypes was feasible in mammals only by exploiting homologous recombination technology in mice [77]. More recently, germ-line competent embryonic stem cells have also been established in rat, so that homologous recombination can now be performed in this species as well [78]. The development of genome editing approaches, using engineered zinc finger nucleases, opened up the possibility to extend such analyses to other organisms and tissue culture cells [79]. Still, a gene knock-out is laborious and, if the work is given to a contractor, expensive. Many miRNAs are located in introns of protein coding genes; so knock-out of a miRNA can result in simultaneous inactivation of the “host” protein coding gene with potentially detrimental effects that are unrelated to miRNA action. Finally, as discussed below, it is often desirable to downregulate the function of several miRNAs together, which is not feasible through knock-out. Interference with multiple miRNAs that are not synthesized from a single polycistronic gene is much easier to achieve using a knock-down approach.

The knock-down of miRNAs can be performed by application of antisense oligonucleotides (ASOs). Usually, ASOs exert their effects independently of the cellular silencing machinery: their potential in biological assays was described as early as 1978 [80]. Since then, lot of experience has been gained in the design of stable, specific, and potent antisense reagents for research and therapeutic use. Since unmodified oligonucleotides are quickly degraded by nucleases when administered to cells, chemical modification is necessary to enhance stability and potency. (For a review on chemical modification used in antisense approaches, see [81].) Inactivation of complementary RNA can be achieved, for example, by delivery of DNA oligonucleotides. RNA/DNA hybrids are then recognized and cleaved by RNase H [82]. Alternatively, oligonucleotides can be coupled to ribozymes or DNazymes that cleave bound RNA by cleavage [81]. For the inactivation of miRNAs, RNA-based oligonucleotides that do not carry enzymatic activity have gained popularity. It is not clear whether these oligonucleotides work by inducing degradation of the targeted miRNA [83] or by forming stable ASO/miRNA heteroduplexes and, thereby, blocking miRNA function [84, 85].

ASOs and ribozymes that interfere with miRNA function are commonly referred to as antagoniRs and antagomiRzymes, respectively, and can be obtained from commercial sources. In addition, plasmid- and virus-derived vectors exist that both allow for simultaneous and permanent expression of antagoniRs targeting different miRNAs and permit measurement of expression levels by coexpression of a fluorescent reporter protein [86].

In principle, miRNA function can be inhibited at different stages [87]. For example, ASOs have been used to interfere with the maturation of pri-miRNA [88]. Targeted degradation of pri-miRNAs in the nucleus with RNase H-based ASOs has also been tried [87]. Strategies to target pri-miRNAs could have the advantage of combined inhibition of several miRNAs transcribed from the same polycistronic gene locus. Experiments with siRNAs targeting the loop region of pre-miRNA have been reported [89], but the approach has not acquired much popularity: the loop region might be difficult to access, leading to inefficient knock-down of miRNA [87].

With regard to knock-down of protein-coding mRNAs, siRNA are nowadays in much wider use than ASOs, probably because siRNAs provide a more potent and efficient mode
<table>
<thead>
<tr>
<th>Aim</th>
<th>Method</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knock-out of miRNA gene</td>
<td>Homologous recombination/Gene editing with zinc finger nucleases</td>
<td>(i) Precise intervention</td>
<td>(i) Laborious and time consuming</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Complete loss-of-function</td>
<td>(ii) Simultaneous knock-out of protein encoded by the same transcriptional unit</td>
</tr>
<tr>
<td>Knock-down of miRNA</td>
<td>Antisense oligonucleotide Ribozymes/DNAzymes</td>
<td>(i) Can attack mature miRNA and miRNA precursors</td>
<td>(i) OTEs and unspecific secondary effects may occur</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Stable expression from vector-constructs</td>
<td>(ii) Efficiency of knock-down is difficult to predict</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iii) Commercially available</td>
<td>(iii) Multiple knock-downs might be needed to produce a phenotype</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iv) Transfection/transduction of multiple oligonucleotides is possible</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miRNA sponges</td>
<td>(i) Easy design</td>
<td>(i) Efficiency of knock-down is difficult to predict</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Simultaneous knock-down of multiple miRNAs</td>
<td>(ii) Incomplete knock-down of individual miRNAs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iii) Expression can be verified by fluorescent reporter</td>
<td></td>
</tr>
<tr>
<td>Over-expression of miRNA</td>
<td>miRNA mimics</td>
<td>(i) Efficient silencing of target mRNA</td>
<td>(i) Oversaturation of RNAi machinery can lead to secondary effects</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Oligonucleotides mimicking mature miRNAs or miRNA precursors could be used</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iii) Transfection/transduction of multiple mimics is possible</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Conditional release of miRNA from riboswitch constructs</td>
<td>(i) Controlled expression of mature miRNA</td>
<td>(i) Laborious design</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Possibly less toxic side effects</td>
<td>(ii) Lack of availability</td>
</tr>
<tr>
<td></td>
<td>Target protectors</td>
<td>(i) Release of specific mRNA from regulation by miRNA possible</td>
<td>(iii) Not yet adaptable to high-throughput analysis</td>
</tr>
<tr>
<td>Release of target mRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
of intervention. In some model organisms, like zebrafish, however, a certain class of ASOs, called “morpholinos”, is still indispensable for knock-down of gene expression and so far could not be replaced by siRNA-based approaches [90].

More recently, vector constructs have been devised that contain multiple binding sites for miRNAs, in order to compete with endogenous mRNAs for miRNA binding. Such miRNA sponges appeared to be a versatile tool for miRNA research [91]. Loya and colleagues analysed phenotypes when miR-7, miR-8, and miR-9a in Drosophila were down-regulated by miRNA sponges. The authors found that miRNA sponges evoked the same phenotype as produced by homologous recombination, but in a milder form. This is expected: miRNA sponges do not completely sequester target miRNAs, because of the competitive binding of miRNAs to miRNAs. Still, this approach enables not only simultaneous down-regulation of several miRNAs but also inducible expression of miRNA sponges in specific tissues and at different time points, allowing spatiotemporal analysis of miRNA function. For instance, it was possible to assign the known role of miR-8 in neuromuscular junction formation by expression of miRNA sponge that targeted miR-8 in muscle cells [92].

As an alternative to, or to complement, loss-of-function studies, miRNA-mimics can be used to achieve de novo or enhanced expression of miRNA (gain-of-function). miRNA-mimics can be administrated as synthetic small dsRNA with sequences identical to those of endogenous miRNAs [93, 94]. They resemble siRNA molecules and are readily incorporated into the cellular RNA silencing machinery. If stable ectopic expression of miRNA is desired, constructs are at hand into the cellular RNA silencing machinery, but in a milder form. This is expected: miRNA sponges do not completely sequester target miRNAs, because of the competitive binding of miRNAs to miRNAs. Still, this approach enables not only simultaneous down-regulation of several miRNAs but also inducible expression of miRNA sponges in specific tissues and at different time points, allowing spatiotemporal analysis of miRNA function. For instance, it was possible to assign the known role of miR-8 in neuromuscular junction formation by expression of miRNA sponge that targeted miR-8 in muscle cells [92].

Spatial and temporal regulation of miRNA expression can also be attained by allosteric ribozymes, riboswitches [96]. These are modular constructs containing an aptamer domain (an RNA sequence that specifically binds a chemical compound) embedded within a ribozyme and fused to a pri-miRNA analogue. This construct is functionally inactive in the absence of the appropriate chemical trigger. Upon application of the trigger substance (e.g., theophylline in the case of the cited work) a conformational change is induced in the ribozyme leading to its activation. The ribozyme will then cleave the modular RNA in cis, with pri-miRNA released from the construct and processed [96]. The large number of known aptamers and their endogenous and exogenous chemical triggers could develop this approach of conditional RNA interference into a versatile alternative for inducible vector constructs, especially if toxic side effects in knock-down or knock-out approaches are of concern [97].

Finally, specific mRNAs can be released from miRNA-mediated inhibition by application of target protectors. In experiments performed by Choi et al. in zebrafish [98], morpholino-based ASOs were used, which prevented miR-430 binding by blocking seed-matched sites and neighbouring nucleotides in two target RNAs. In this way, the authors succeeded in specifically interfering with miR-430-mediated translational repression. To conduct this type of experiment, it is necessary to know the targets of the miRNA of interest, but target protectors can be valuable in target validation and in characterization of miRNA: mRNA interactions.

6. miRNA-Based Functional Screens

The established infrastructure for siRNA/shRNA screens (i.e., robotics for large-scale sample preparation, automated data acquisition and analysis, data storage capacities) can easily be applied for high-throughput studies of miRNA function. As for siRNA-based screens, lipid-based transfection is most commonly used to achieve a transient overexpression [99] or inhibition [100] of miRNAs in cell culture. A number of screens were completed under conditions of miRNA stable overexpression [40, 45, 101, 102], achieved by transduction with retroviral vectors encoding specific miRNA genes [40]. Alternatively, constitutive overexpression or down-regulation of non-coding RNAs can be obtained by adenoviral vector-based systems [69, 103]. The advantages of the reverse transfection method, coupled with an automatic liquid handling system, have been also utilized in miRNA-based screens by several groups with a high success rate [104, 105].

The first miRNA was described in the early 1990s [50], but the initial accumulation of data concerning regulatory roles was rather slow [106–108]. This in turn delayed the availability of reagents to modulate the activity of endogenous miRNAs, and so the first functional miRNA-based screen [100] was completed a couple of years later than siRNA-based screens [109]. As a consequence, though, miRNA-based screens could benefit a lot from prior experience accumulated with siRNA-based screens.

7. Biological Processes Analysed in miRNA-Based Screens

A number of miRNA-based screens have been completed during the last 5 years (Table 2). Two major groups of biological processes have been investigated so far, namely, (i) cell viability, proliferation, and apoptosis and (ii) gene transcription and/or activity regulation.

Numerous miRNAs were shown to inhibit (let-7, miR-34a, miR-143, miR-145, miR-221, miR-222) [117–121] or to stimulate (miR-21, miR-133) [122, 123] cell proliferation. Moreover, recent studies demonstrated that a well-studied cluster of miR-17-92 can act both as oncopgenes [124–127] and as tumour suppressors [9, 128], and these roles are probably cell type- and/or environment-dependent. Context-dependent activity was also reported for miR-24: down-regulation of miR-24 inhibited proliferation of A549 cells but increased growth of HeLa cells [100].

A considerable increase in the numbers of miRNAs that regulate cell proliferation and apoptosis was obtained...
<table>
<thead>
<tr>
<th>Model system</th>
<th>Number of miRNAs screened</th>
<th>Type of regulation</th>
<th>Phenotype measured</th>
<th>Assay</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa and A549</td>
<td>95</td>
<td>Loss-of-function by miRNA inhibitors</td>
<td>Cell proliferation and apoptosis</td>
<td>Cell counting and caspase-3/7 activity assay</td>
<td>2005</td>
<td>[100]</td>
</tr>
<tr>
<td>Primary hTERT-immortalized BJ-EHT fibroblasts</td>
<td>~450</td>
<td>Gain-of-function by stable miRNA expression</td>
<td>Sustained proliferation</td>
<td>miR-Array</td>
<td>2006</td>
<td>[40]</td>
</tr>
<tr>
<td>HeLa</td>
<td>~450</td>
<td>Gain-of-function by stable miRNA expression</td>
<td>p27&lt;sup&gt;Kip1&lt;/sup&gt; regulation</td>
<td>GFP reporter assay and miR-Array</td>
<td>2007</td>
<td>[45]</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>187</td>
<td>Gain-of-function by miRNA precursors</td>
<td>TRAIL-induced caspase-3 activation</td>
<td>Caspase-3/7 activity assay</td>
<td>2007</td>
<td>[104]</td>
</tr>
<tr>
<td>Drosophila clone 8 cells</td>
<td>77/78 miRNA loci</td>
<td>Gain-of-function by plasmid-based miRNA expression</td>
<td>Wg signaling pathway regulation</td>
<td>Luciferase reporter assay</td>
<td>2007</td>
<td>[110]</td>
</tr>
<tr>
<td>Neuroblastoma cell lines</td>
<td>8</td>
<td>Gain- and loss-of-function by miRNA mimics and inhibitors</td>
<td>Proliferation</td>
<td>Change in electrical impedance</td>
<td>2008</td>
<td>[111]</td>
</tr>
<tr>
<td>MCF7</td>
<td>~450</td>
<td>Gain-of-function by stable miRNA expression</td>
<td>Cell migration</td>
<td>Trans-well cell migration assay</td>
<td>2008</td>
<td>[101]</td>
</tr>
<tr>
<td>HeLa</td>
<td>91</td>
<td>Gain-of-function by miRNA mimics</td>
<td>p53 gene activity</td>
<td>Luciferase reporter assay</td>
<td>2009</td>
<td>[38]</td>
</tr>
<tr>
<td>HeLa</td>
<td>~450</td>
<td>Gain-of-function by stable miRNA expression</td>
<td>Per gene family regulation</td>
<td>GFP reporter assay and miR-Array</td>
<td>2009</td>
<td>[102]</td>
</tr>
<tr>
<td>Primary ovarian granulosa cells</td>
<td>80</td>
<td>Gain-of-function by miRNA precursors</td>
<td>Progesterone, testosterone and estradiol release</td>
<td>Enzyme immunoassay (EIA)</td>
<td>2009</td>
<td>[112]</td>
</tr>
<tr>
<td>HEK 293</td>
<td>266</td>
<td>Gain-of-function by miRNA mimics</td>
<td>p21Cip/Waf1 regulation</td>
<td>Luciferase reporter assay</td>
<td>2010</td>
<td>[46]</td>
</tr>
<tr>
<td>HCT-16</td>
<td>810</td>
<td>Gain-of-function by miRNA mimics</td>
<td>Cell viability in the presence of Bcl-2 family inhibitor ABT-263</td>
<td>CellTiter-Glo Luminescent Cell Viability Assay</td>
<td>2010</td>
<td>[99]</td>
</tr>
<tr>
<td>HEK 293T</td>
<td>107</td>
<td>Gain-of-function by miRNA mimics</td>
<td>p53 gene regulation</td>
<td>Luciferase reporter assay</td>
<td>2010</td>
<td>[39]</td>
</tr>
<tr>
<td>HCT116 p53&lt;sup&gt;+/+&lt;/sup&gt;, H460 and MCF7</td>
<td>5</td>
<td>Gain-of-function by miRNA precursors</td>
<td>p53 gene regulation</td>
<td>Western blot</td>
<td>2010</td>
<td>[10]</td>
</tr>
<tr>
<td>Huh-7</td>
<td>327</td>
<td>Gain-of-function by miRNA precursors</td>
<td>Lipid droplet formation and growth</td>
<td>Immunocytochemistry and fluorescence microscopy</td>
<td>2010</td>
<td>[105]</td>
</tr>
<tr>
<td>Primary ovarian granulosa cells</td>
<td>80</td>
<td>Gain-of-function by miRNA precursors</td>
<td>Proliferation and apoptosis</td>
<td>Immunocytochemistry and fluorescence microscopy</td>
<td>2010</td>
<td>[113]</td>
</tr>
<tr>
<td>HMEC</td>
<td>328</td>
<td>Gain-of-function by miRNA precursors</td>
<td>Proliferation</td>
<td>Fluorescence microscopy</td>
<td>2010</td>
<td>[114]</td>
</tr>
</tbody>
</table>
Table 2: Continued.

<table>
<thead>
<tr>
<th>Model system</th>
<th>Number of miRNAs screened</th>
<th>Type of regulation</th>
<th>Phenotype measured</th>
<th>Assay</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIA PaCa-2</td>
<td>445</td>
<td>Gain-of-function by stable miRNA expression</td>
<td>Proliferation</td>
<td>Custom-made microarray</td>
<td>2010</td>
<td>[115]</td>
</tr>
<tr>
<td>HeLa and HeLa P4</td>
<td>8</td>
<td>Gain- and loss-of-function by miRNA precursors and inhibitors</td>
<td>Cell proliferation and trafficking</td>
<td>Fluorescence microscopy</td>
<td>2011</td>
<td>[116]</td>
</tr>
</tbody>
</table>

since Cheng et al. reported the first large-scale screen to identify mammalian miRNAs involved in these processes [100]. In 2007, Ovcharenko et al. performed a screen with 187 synthetic oligonucleotides to capture the modulators of TRAIL-induced apoptotic pathway [104]. They found that 34 of tested miRNAs modulate the activity of caspase-3. Recently, a gain-of-function miRNA-based screen was completed in human colorectal cancer DLD-1 cells [11]. By measuring cell viability, authors not only confirmed function of already known oncogenic miR-372 and miR-373 [40] but also discovered novel miRNAs involved in cell proliferation and apoptosis. miR-491 was among the strongest antiproliferative miRNAs, and further experimental analysis revealed that it induces apoptosis via direct down-regulation of antia apoptotic Bcl-xL [129]. miRNAs regulating expression of another member of Bcl-2 protein family, Mcl1, were identified by screening a library of 810 human miRNAs for the ability to confer resistance of cancer cells to ABT-263, an inhibitor of Bcl-2 family members [99]. Viability measurements of human colorectal cancer HCT-16 cells transiently transfected with miRNA mimics revealed 19 miRNAs that sensitized cells to ABT-263. 15 of these miRNAs showed the same phenotype in melanoma CHL1 cells. Furthermore, 10 out of 12 strong sensitizer miRNAs that were analysed for targeting 3′ URT of Mcl1 were confirmed as direct regulators of the gene. These examples of the screens demonstrate that miRNAs that modulate sensitivity to chemotherapeutic agents can be identified and potentially, in future, used in cancer therapy [21, 130]. The feasibility of such screens has vastly improved by the evolution of methods to quantify cell proliferation from straightforward cell counting [100] to recording of electrical impedance over 96 hours [111].

miRNAs acting on transcription and/or gene activity regulation are usually identified in so-called “target-based screening”. In the most cases, luciferase or green fluorescent protein (GFP) is fused to the gene of interest and the strength of the detected signal is used to gauge the expression or activity of the test gene [38, 39, 45, 46, 102]. Using a functional genetic approach with stable expression of individual miRNAs [40], miR-221 and miR-222 were demonstrated to specifically regulate expression of tumour suppressor p27Kip1 [45], miR-192/194 cluster-modulate expression of Per gene family [102].

The repertoire of cellular processes analysed by miRNA functional screens is expanding rapidly (Table 2). Doing miRNA-based screens in appropriate cellular contexts, for instance, for miRNAs regulating steroidogenesis in ovarian cells [112], and for miRNAs regulating lipid droplet formation in hepatocytes [105] helps to ensure acquisition of physiologically relevant information.

8. Gain-of-Function versus Loss-of-Function in miRNA-Based Screens

Although reagents for gain-of-function and loss-of-function miRNA experiments are available to similar extents, nearly all reported screens to assess the function of miRNAs utilized the gain-of-function approach (Table 2). This trend probably arose because of the ease with which ectopic expression of miRNAs can be confirmed. For instance, exogenous reporter gene assays [116, 131], qRT-PCR, and Northern blotting and ribonuclease protection assay [40] are easily applicable methods to measure over-expression of miRNAs. In contrast, the only assays that have been extensively used so far to show the inhibition of endogenous miRNA activity have involved reportors [38, 100, 128, 132]. The popularity of gain-of-function screens might additionally be explained by potentially easier evaluation. Over-expression of miRNAs might induce accentuated phenotypes, which might be not related to the levels of endogenous miRNAs, whereas the evaluation of data obtained under the conditions of miRNA down-regulation is possible only then one knows expression level of endogenous miRNAs in the test system. Acquisition of these data sets, therefore, needs thorough additional experimentation.

9. Fluorescence Screening Microscopy for miRNA-Based Screens

There are virtually no reasons why the read-out strategies in miRNA-based screens should be different from the ones established in siRNA-based screens. Nevertheless, we consider fluorescence microscopy screens to be highly advantageous. Features that make such microscopy ideal to analyse regulatory potential of miRNAs include the following: (i) rapid collection of large amount of data, (ii) feasibility of phenotype multiplexing, (iii) the possibility to acquire quantitative data on a cell-by-cell basis and/or population-based basis, and (iv) detection of subtle phenotypes [133, 134]. One of the first studies applying this
technology for functional miRNA studies was by Sirotkin and colleagues, who reported an immunocytochemistry- and fluorescence-microscopy-based screen to identify miRNAs regulating proliferation and apoptosis [113]. Primary human ovarian cells were transfected with synthetic miRNA precursors, and PCNA and cyclin B1 proteins were used as markers of proliferation. The expression levels of Bax and caspase-3, in combination with the TUNEL assay, were used to determine the extent of apoptosis. The power of technology is demonstrated by another microscopy-based screen: using automated image analysis and nucleus classification software, a novel antiproliferative activity of miR-320a was discovered [116]. The screen to identify miRNAs controlling lipid droplet formation in hepatocytes [105] illustrated the sensitivity of fluorescence microscopy-based approach, which was comparable to the laborious biochemical assay—11 out of 327 transiently overexpressed human miRNAs were selected via an automated work flow as the most potent regulators of intracellular lipid content [105].

10. Validation of Hits in miRNA-Based Screens

In current screens, the list of primary hits involved in regulation of a biological pathway can be obtained reasonably fast, if necessary infrastructure is on place. The primary hit lists in siRNA/shRNA-based screens are usually validated by (i) repeating the assay with different types of reagents, and (ii) secondary assays, and (iii) rescue experiments, in which a phenotype caused by knock-down of a certain gene product is rescued by the over-expression of a construct that cannot be attacked by siRNA (either due to mutation or because of origin from another species). Currently, there is no clear agreement on criteria for successful validation of primary hit miRNAs, except from reproducing the phenotype with different reagents. Additionally, the effects caused by miRNA over-expression and down-regulation could be compared. Since this can be laborious, lists of miRNAs that cause a particular phenotype in screens may usefully be published without further analysis [100, 112, 113], allowing follow-up by others.

In order to perform a follow-up research on hit miRNAs, prediction of their target genes needs to be done first. Many computational algorithms have been developed over the last decade to predict miRNA targets [135], and a combination of multiple algorithms is frequently used to narrow down the candidates. Interesting strategy was used in the studies of TRAIL-induced apoptosis when hits of miRNA-based screen were compared with those of siRNA-based screen in order to identify plausible interactions between miRNAs and their target mRNAs [104].

Many of the screens that have been completed so far have been extended to experimental identification and validation of the targets of the hit miRNAs (Table 3). The most widely used method to test a direct regulation of gene expression by miRNAs is based on reporter assays. The 3′UTR, the 5′UTR, or the whole gene is cloned immediately downstream of a reporter gene encoding luciferase or fluorescent protein. The construct is then transiently cotransfected with miRNA mimics or antimiRs into host cells and luciferase activity or fluorescence is measured after 24–48 hours of incubation [10, 11, 38, 99, 110]. Additional evidence that the target gene is directly regulated by an miRNA can be provided by mutating [38, 110] or deleting [10, 101] predicted miRNA binding sites in the 3′UTR of a reporter vector. As controls, the entire 3′UTR can be inserted in the reverse orientation [136] or truncated [110]. Frequently, qRT-PCR has been used to show degradation of target mRNAs in miRNA overexpressing cells [10, 45, 46, 101].

Since miRNAs can execute their regulatory action by repressing translation and/or promoting mRNA decay of target genes [137], measurement of the product protein level is an additional means to validate miRNA action [11, 45, 46]. Biochemical techniques for the isolation of target mRNAs were also shown to be rewarding [138–140]. Eventually, the action of miRNA can be tested by down-regulation of known or putative target genes and monitoring the resulting phenotypes [72, 105]. Observation of the same phenotype after both miRNA over-expression and down-regulation of its putative target genes strengthens evidence that the target identification was valid. Such tests might include down-regulation of a single target as well as multiple ones. Complex evaluations are possible if combining several validation approaches. For example, changes in target protein level without any changes in a reporter assay would suggest that the miRNA does not directly target an affected gene but rather regulates its modulator or the whole pathway [99].

11. Challenges of siRNA-, shRNAs-, and miRNA-Based Screens

RNAi-based screens have turned to be extremely useful for studies in various fields of biology and medicine [59, 141–143]. A robust assay, careful design of the experiment, reliable controls, and exhaustive testing of the reagents are crucial for successfully completing a screen, and several excellent reviews, describing the planning of RNAi screen experiments, have been published (e.g., [60, 144–146]). We will now focus on some practical aspects of siRNA/shRNA-based screens that could be relevant while performing miRNA-based screens.

The analysis of siRNA-based screens is often complicated by off-target effects (OTEs): phenotypes caused by unspecific down-regulation of mRNAs. One source of OTEs is partial homology of siRNAs with unintended mRNAs. A different type of OTE arises from induction of cytokine production by siRNA/shRNA with certain sequence motifs [147], which ultimately results in apoptosis. Therefore, in the last years considerable effort has been invested in improving the performance of siRNAs/shRNAs. For example, induction of an interferon response can be mitigated by excluding G-U-rich sequence motifs in siRNA molecules [148]. Specificity can be considerably enhanced by altering the chemical backbone of siRNAs: examples are LNA (locked nucleic acid) nucleotide analogues or modifying ribosyl groups by addition of a 2′-O-methyl group at specific positions [149, 150].
Table 3: Validation assays in miRNA-based screens.

<table>
<thead>
<tr>
<th>Effector miRNA</th>
<th>Target genes</th>
<th>Expression</th>
<th>Validation assay</th>
<th>IS</th>
<th>siRNA</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-372 and -373</td>
<td>LATS2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2006</td>
<td>[40]</td>
</tr>
<tr>
<td>miR-221 and -222</td>
<td>p27Kip1</td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
<td>2007</td>
<td>[45]</td>
</tr>
<tr>
<td>miR-315</td>
<td>Axin and Notum</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>2007</td>
<td>[110]</td>
</tr>
<tr>
<td>miR-34a</td>
<td>Bcl2 and MYCN</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>2008</td>
<td>[111]</td>
</tr>
<tr>
<td>miR-373 and -520c</td>
<td>CD44</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>2008</td>
<td>[101]</td>
</tr>
<tr>
<td>miR-29b,c</td>
<td>p85α and CDC42</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>2009</td>
<td>[38]</td>
</tr>
<tr>
<td>miR-192 and -194</td>
<td>Per1, 2 and 3</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>2010</td>
<td>[102]</td>
</tr>
<tr>
<td>28 miRNAs</td>
<td>p21Cip/Waf1</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>2010</td>
<td>[46]</td>
</tr>
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<td>10 miRNAs</td>
<td>MCL1</td>
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<td>+</td>
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<td>+</td>
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<td>[39]</td>
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<tr>
<td>miR-504</td>
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<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>2010</td>
<td>[10]</td>
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<tr>
<td>11 miRNAs</td>
<td>Multiple genes</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>2010</td>
<td>[105]</td>
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<tr>
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<td>+</td>
<td>+</td>
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<td>[11]</td>
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<tr>
<td>28 miRNAs</td>
<td>p21Cip/Waf1</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>2010</td>
<td>[114]</td>
</tr>
</tbody>
</table>

a: mRNA expression profiling.
b: Luciferase and/or GFP reporter assays.
c: Immunostaining.
d: Recapitulation of miRNA-mediated phenotype by selected siRNAs.

Secondary or even toxic effects caused by transfection reagents alone and/or in combination with silencing RNA cannot be completely ruled out. Fortunately, current formulations contain 5 to 10 times less reagents than were required several years ago. Another approach is to pool several siRNAs against the same target, while using reduced amounts of each. The idea behind this approach is that the target mRNA will be attacked by all, or the majority of, siRNAs in the pool, and the specific down-regulations will act cumulatively, whereas OTEs of individual siRNAs will be too weak to affect results. The same concept of improved signal-to-noise ratio in observed phenotypes forms also the basis for the application of esiRNAs [65].

Despite these strategies, though, OTEs and unspecific secondary effects cannot be avoided completely. It is, therefore, essential to control for both. One option is to use several different siRNAs to target the same mRNA at independent sites. The probability that an observed phenotype corresponds to an on-target silencing effect rises with the number of siRNAs causing the same phenotype [151]. Moreover, it is of advantage to measure several cellular parameters (e.g., cell shape and size, number of cells) in a screen. Multiparametric analysis allows generation of phenotypic profiles of individual siRNAs. A high degree of overlap in these profiles justifies strong confidence in the specificity of an observed phenotype [58, 151].

One of the most serious concerns in functional screens is possible oversaturation of the cellular silencing machinery by exogenous siRNA/shRNA and miRNA mimics. This can result in derepression of mRNAs that are regulated by endogenous miRNAs [152, 153]. Various strategies have been developed to deal with these problems (for a detailed compilation of strategies see [154]). Briefly, overloading the cellular RNA silencing machinery can be avoided by applying the lowest possible amounts of siRNA/shRNA to the cells. For instance, transcription from RNA Pol II-driven promoters, and use of inducible constructs, can be useful to control levels of vector-encoded shRNAs [70, 155]. Using vectors derived from adeno-associated virus can also be a way to induce a moderate level of stable shRNA expression in cells [21, 156]. Coexpression of AGO-2 has also been described as an effective approach to overcome the problem of saturating the RNAi machinery by ectopic siRNA, shRNA, and miRNA [157]. Enhanced amounts of AGO-2 were shown to drive RNAi interference toward preferential knock-down of perfectly matched target mRNAs in diverse mammalian cell lines. Because, in addition, less siRNA/shRNA was needed for specific knock-down, and unspecific targeting of miRNAs was significantly reduced in the presence of ectopic AGO-2, the authors suggest that this strategy might result in reduced rates of false negatives and false positives in RNAI-based screening approaches. On the other hand, the suitability of the approach should be tested rigorously, as AGO-2 was recently shown to play a role in biogenesis of particular type of miRNAs [36, 37].

Finally, miRNA-based functional screens might suffer from neutral repression, when reduced transcript levels do not lead to measurable changes in cellular behaviour. Sometimes, residual protein is still sufficient to fulfil the required function; alternatively, feedback mechanisms exist that compensate functionally for the knock-down [158].

Usually, screens encompass thousands of genes or even the whole genomes; so it is hardly possible to look for functions that might be conferred by redundant gene function. Often, cellular functions are not controlled by just one protein, but by two or more isotypes or evolutionary-related forms of proteins to ensure higher reliability of the process, and metabolic pathways may be redundant.
The need to use several siRNAs per protein, and multiple possible combinations of distinct siRNAs, limits studies that aim to identify redundant gene products to relatively small, directed screens. Conversely, highly homologous gene products, like, for example, splice variants, might have distinct cellular functions, but siRNAs might target all forms together (e.g., [159]). Similar concerns pertain to miRNA-based screens, as redundant activities of miRNAs in regulation of many biological processes is well documented [160]: Miska et al. reported that most miRNAs in C. elegans are individually dispensable [161], and Voorhoeve et al. found that miR-372 and miR-373 cooperate with oncogenic RAS in the development of testicular germ cell tumours [40]. A way to deal with miRNA redundancy could be the use of sensitized genetic backgrounds as described by Brenner et al. [162]. By deleting one of the two AGO genes in C. elegans the authors partially disabled the RNA silencing machinery. In addition, worms with defects in chromatin modification or transcriptional regulation were generated. Using these organisms allowed definition of biological roles for several individual miRNAs [162]. Additionally, synthetic phenotypes might be used to analyse functions of miRNAs [160].

The complexity of miRNA action presents a challenging task for high-throughput functional analysis. However, given our experience from RNAi screens over the last decade, with siRNAs and shRNAs in different model organisms, careful experimental design and exhaustive target validation makes this powerful technology indispensable for understanding the biological roles of miRNAs.

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R. Nagel, L. Clijsters, and R. Agami, “The miRNA-192/194

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W. Y. Choi, A. J. Giraldez, and A. F. Schier, “Target protectors

D. Kumar, C. I. An, and Y. Yokobayashi, “Conditional RNA


K. Schmidt-Supprian and K. Rajewsky, “Vagaries of condi-

W. Y. Choi, A. J. Giraldez, and A. F. Schier, “Target protectors

L. T. Lam, X. Lu, H. Zhang, R. R. Lesniewski, S. H. 

R. Nagel, L. Clijsters, and R. Agami, “The miRNA-192/194

A. E. Pasquinelli, B. J. Reinhart, E. Slack et al., “Conser-

P. Aza-Blanc, C. L. Cooper, K. Wagner, S. Batalov, Q. L. 

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Review Article

Molecular Beacons: Powerful Tools for Imaging RNA in Living Cells

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Recent advances in RNA functional studies highlights the pivotal role of these molecules in cell physiology. Diverse methods have been implemented to measure the expression levels of various RNA species, using either purified RNA or fixed cells. Despite the fact that fixed cells offer the possibility to observe the spatial distribution of RNA, assays with capability to real-time monitoring RNA transport into living cells are needed to further understand the role of RNA dynamics in cellular functions. Molecular beacons (MBs) are stem-loop hairpin-structured oligonucleotides equipped with a fluorescence quencher at one end and a fluorescent dye (also called reporter or fluorophore) at the opposite end. This structure permits that MB in the absence of their target complementary sequence do not fluoresce. Upon binding to targets, MBs emit fluorescence, due to the spatial separation of the quencher and the reporter. Molecular beacons are promising probes for the development of RNA imaging techniques; nevertheless much work remains to be done in order to obtain a robust technology for imaging various RNA molecules together in real time and in living cells. The present work concentrates on the different requirements needed to use successfully MB for cellular studies, summarizing recent advances in this area.

1. Introduction

A wealth of experimental evidence accumulated to this date illustrates the wide range of cellular functions conducted by ribonucleic acids (RNAs). In many occasions these functions involve RNA transport from one cellular compartment to another or into the same compartment to specialized regions. In order to accomplish this, RNA molecules are under a specific and selective control via expression levels and/or stability in a spatial-temporal manner. Different methods developed to purify RNA [1–3] from cell populations or tissues have provided relevant information about the relative concentration of RNA in cells, or cellular compartments, yet these methods provide limited information about the spatial-temporal distribution of RNAs and their dynamic transport.

The integral understanding of cellular process in which RNA is involved, requires a method that reveals RNA localization in real time in a subcellular context in living cells. The information obtained from this type of assays promise to impulse the advancement in molecular biology, medical research, and diagnostics.

Many methods have been developed to measure RNA expression levels between different cell populations, such as the polymerase chain reaction (PCR) [4], northern blot [5], serial analysis of the gene expression (SAGE) [6], suppression of subtractive hybridization (SSH) [7], differential display [8], representational difference analysis (RDA) [9], expressed sequence tag (EST) [10], and microarrays [11]. Thus these techniques in addition to databases containing genomic data for a wide variety of biological entities, supply a useful instrument for understanding physiology or pathology at the molecular level. Nevertheless, none of the techniques previously mentioned could provide information about the subcellular RNA localization and its transport.

Through the use of fluorescent in situ hybridization assays [12], scientists have identified gradients of RNA in cells [13], embryos [14], and tissues [15, 16]. However this technique is conducted on fixed samples and therefore provides limited information about the dynamics of such
gradients. Other disadvantages are the laborious and time-consuming nature of the assay, the difficulty that involves the analysis of the images and variations due to handling different trials. The chemicals used for dehybridization and fixation could affect the signal level [17] and alter the integrity of organelles, hampering any conclusions about spatial changes in organelles or compartments.

The most adequate procedure to obtain spatial-temporal resolution of the RNA dynamics is using living cell assays. Under these conditions, the probes to be used must have high specificity, sensitivity, and good signal to background ratio, especially for low abundance RNA molecules. Ideally, the experimental procedure should be able to detect discrete changes in RNA concentrations of the sample. Furthermore, a robust method should provide additional information like single nucleotide mutations, deletions, insertions, and single nucleotide polymorphisms (SNPs). Another fact to take into consideration is the method for intracellular delivery; it should not produce the degradation of the probe or damage the cell. Finally, the probes utilized should have fast kinetics of hybridization with its target at room temperature, in order to be useful in real-time measurements.

Recent advances in DNA chemistry deliver promising nanostructured probes known as molecular beacons (MB), which may fulfill many (if not all) of the requirements highlighted above. MBs are slowly becoming powerful tools to explore RNA function and dynamics in living cells. Combined with advanced imaging techniques, MB are delivering interesting and sometimes surprising results about RNA dynamics in living cells and tissues.

A recent work reviews different forms of linear and nonlinear probes useful for cell imaging studies [18]. In this review we will address the use of MBs for RNA imaging in living cells, analyzing some of the key features required for successful MB design. We will review also various delivery methods commonly used for MB introduction into living cells.

2. Structure and Function of Molecular Beacons

MBs are oligonucleotide probes that fluoresce upon hybridization with its targets [20]. To build a MB, an oligonucleotide chain is linked covalently to a fluorescent dye (reporter) usually at 5’ end and to a fluorescence quencher at the opposite end. The probes have the capability to produce a stem-loop hairpin structural conformation in absence of its target, due to complementary sequences from 5 to 7 nucleotides in length, located at the termini of the molecule (Figure 1(a)). The resulting secondary structure of the MB brings together the reporter and the quencher, making possible the energy transfer, which produces fluorescence quenching. When MB hybridizes to its target, the reporter and quencher are separated (≥10 nm), preventing the quenching of the fluorescence from the reporter (Figure 1(b)).

Most MB studies have been carried out using nucleic acids in in vitro assays. In fact, several real-time PCR strategies involve the use of MB for monitoring sample amplification [21–25]. Such strategies involve heating of the solution to high degrees in order to maximize MB-target association. Unfortunately, heating to high temperatures cannot be conducted when studying living cells, and therefore MB design should account for MB-target association at physiological temperature.

We have found that a careful design of the MB stem sequence is essential for MB-target association at physiological temperature ranging from 20 to 37 degrees centigrade.

In a solution of MB containing an excess of target (≥fivefold MB concentration) it is possible to observe an increment in fluorescence intensity between 10- and 200-fold [20] in comparison with a solution without a target (Figure 1(c)). When target concentration is unknown (like in the case of nucleic acids detection in living cells) it is desirable to have fast hybridization rates and improved signal to background ratio, especially when the target is a nucleic acid present in a low concentration. Some MB design strategies have been implemented recently to address this issue.

MB shows an increased specificity in comparison to linear probes having an equal number of complementary nucleotides with its target; this is due to competition between the stem-loop hairpin structure and the MB-target hybridized state [26]. This phenomenon gives the capability to the MB to discriminate between perfectly matched and single mismatched targets. To reach this high specificity an appropriate MB design is required, taking into consideration the experimental conditions, such as magnesium concentration, ionic strength, and temperature.

We have found that prior to living cell studies, it is highly advisable to conduct hybridization and thermal denaturing assays using a spectrofluorometer and a real-time thermal cycler, respectively. In order to have a prediction about the MB behavior in living cells assays it is recommended to calculate the thermodynamical parameters of the hybridization kinetics, that is, changes in enthalpy and entropy, melting temperature, also association and dissociation constants of a specific MB-target pair using the thermal profile obtained from the thermal denaturing and parameters obtained from the hybridization assays. See [26, 27] for detailed protocols.

Molecular beacons have been used successfully in a wide variety of applications due to its high signal to background ratio, including qPCR [21–25], SNPs detection by real-time PCR [28–31], genotyping [32–36], mutation detection [37–39], assay for nucleic acid cleavage [40, 41], cancer-cell detection [42–44], monitoring viral infection [45–47], and RNA expression, transport, and localization in living cells [48–51].

3. Designing Molecular Beacons

Three key factors must be considered for MB design, the efficiency of the quenching, the stem-loop secondary structure formed by the sequences at the MB termini and the C-G content of the stem (specially relevant for assays conducted at physiological temperatures).

3.1. Reporter and Quencher: Mechanism and Pair Election.

MB use a reporter-quencher pair to build a signal transduction system [52, 53]. The quenching that takes place when
Figure 1: Structure and function of MB. (a) Stem-loop hairpin structure of a MB showing its four structural components: loop, stem, quencher, and reporter. The chemical structure of the linkers is drawn according to the manufacturer (Integrated DNA Technologies, Iowa, USA). (b) Mechanism of MB function. A MB in a solution containing both MB and target could be in three states: free in a stem-loop hairpin conformation, hybridized with target, or unbound in a random-coil conformation. The random-coil conformation of the MB contributes to the background. (c) Fluorescence intensity. The blue line shows the fluorescence intensity for a MB in solution (50 nM) during 400 seconds (background), and the red line corresponds to the addition (100 seconds) of a target oligonucleotide (500 nM) that hybridize the MB at the loop region. An increase in fluorescent intensity of approximately thirteenfold is observed. The reporter is represented with a red circle and the quencher with a blue. UA means arbitrary units.
the MB is in its stem-loop structural conformation (hairpin) occurs by two mechanisms.

The first mechanism is called dynamic quenching and includes Förster’s resonance energy transfer (FRET) and Dexter transfer, also named electron transfer quenching. In the dynamic quenching mechanism, the photon from the reporter is not released to the environment because a long-range dipole-dipole interaction occurs between reporter and quencher. For the dynamic quenching mechanism, the transfer efficiency of the energy depends on the spectral overlap between the emission spectrum of the reporter and the absorption spectrum of the quencher [54], the quantum yield of the donor, the relative orientation (also referred to as dipolar moment), and the distance between the two groups [55]. For a 50% of FRET efficiency, a distance in the range of 20–70 Å (Förster’s distance) is needed between the reporter and quencher, losing all the quenching efficiency at a distance ≥100 Å (10 nm).

The second mechanism called static quenching [56], requires the formation of ground-state complexes and the mechanism depends on the stem sequence, the linkers, and reporter-quencher pair used in the MB. The most important contributor in the quenching phenomenon is the static or contact quenching; the name describes the nature of the process, which occurs only when reporter and quencher are in close proximity, permitting the physical contact between them. Under these circumstances most of the energy transferred is not emitted as light, but emitted in the form of heat through a nonirradiator process [57]. This phenomenon occurs for all the nonfluorescent quenchers and determines basal fluorescence value in the absence of target and the signal-to-noise ratio of the MB. The most frequently used nonfluorescent quenchers in MB synthesis are Dabcyl (4-((4′-(dimethylamino)phenyl)azo)benzoic acid), Black-Hole quenchers BHQ1 and BHQ2, and Iowa Black FQ and RQ. Typical static quenching efficiency for these quenchers is in the range of 85–97% [54, 57]. These quenchers could be paired with various reporters (Table 1). Dabcyl is practically a universal quencher for near-infrared reporters although its absorption peak is around 475 nm. Worth mentioning, this quencher is the least expensive of all.

Most recently, it has been reported that guanine bases can be utilized as quenchers in MB design (a method known as G-quenched MB) [59, 60]. Guanine bases work as quenchers when present at the opposite end of the reporter. The guanine bases could be placed as overhang or not. MBs without overhanged guanines show only a 15% increment in fluorescence intensity moving from the unbound state (in the absence of target) to the target-hybridized form [61], but when using two overhanged guanines a 2-fold is observed [62]. Guanine bases function as quenchers for a variety of reporters, including pyrene, coumarin, acridone, and 6-FAM. Using guanine bases as quenchers improves the yield of MB obtained during the synthesis and reduces significantly its cost. G-quenched MBs have not been used for RNA imaging in live-cell assays, it is a field waiting to be explored. The structure and function of G-quenched MB and other quencher-free probes are widely discussed in reference [63].

### Table 1: Reporter-quencher pairs. For every one reporter is showed the peak of excitation, emission, and also the quenchers that could be used for observing a high quenching efficiency. The excitation peak and emission peak values were obtained from reference [58].

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Excitation peak (nm)</th>
<th>Emission peak (nm)</th>
<th>Compatible quenchers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3</td>
<td>550</td>
<td>605</td>
<td>BHQ-2 and Dabcyl</td>
</tr>
<tr>
<td>Cy5</td>
<td>640</td>
<td>675</td>
<td>BHQ-3 and Dabcyl</td>
</tr>
<tr>
<td>CR-6G</td>
<td>505</td>
<td>570</td>
<td>Dabcyl</td>
</tr>
<tr>
<td>6-FAM</td>
<td>484</td>
<td>525</td>
<td>Iowa Black-FQ, BHQ-1 and Dabcyl</td>
</tr>
<tr>
<td>HEX</td>
<td>525</td>
<td>554</td>
<td>BHQ-1, Iowa Black-FQ and Dabcyl</td>
</tr>
<tr>
<td>TAMRA</td>
<td>555</td>
<td>605</td>
<td>Dabcyl</td>
</tr>
<tr>
<td>TET</td>
<td>490</td>
<td>535</td>
<td>BHQ-1, Iowa Black-FQ and Dabcyl</td>
</tr>
</tbody>
</table>

#### 3.2. Thermodynamics and Design. The MB-target hybridization is due to Watson-Crick basepairing. Experimental evidence (thermal denaturing assays) and binary models that simplify the real behavior of the phenomenon considering only the beginning of the hybridization kinetics and the end (steady-state), support the fact that for a given MB-perfect-matched target pair the melting temperature is 13 ± 3°C higher than for the corresponding single-mismatched target [26, 27, 57]. The capability of a probe to discriminate perfect a perfect matched and single mismatched target is known as selectivity potential, and its magnitude is determined by the difference of the temperatures at the transition phases [64].

Linear probes have just two possible states, one free or unbound and the other bound to a target. Whereas MBs have three possible states, free with stem-loop conformation, free as a random coil and bound to its target (Figure 1(b)). In terms of free energy a MB is most stable in stem-loop conformation (hairpin) than in random coil state; therefore, such transitions must be brief. These suggest that when the temperature increases and the dissociation between the target and the MB occurs, the MB will acquire the stem loop conformation preferentially over the random coil state [65–67]. The previously described phenomenon explains the high signal to background ratio observed with MBs. This suggest also that a more stable stem (due to higher C-G content or stem length) will produce a higher signal to background ratio. MBs with more stable stems would show enhanced
selectivity because the MB will hybridize only when the interactions MB-targets are strong enough to overcome the stem stability. However, a word of caution, MBs with more stable stems may not hybridize to its target at a physiological temperature, preventing its use for cellular studies. Thus a delicate balance between hairpin stability (stem design) and MB-target selectivity must be reached for successful use of MBs in living cell assays.

3.3. Balance between Selectivity and Hybridization Rate. Clearly the greater advantage of MB over linear probes is its enhanced selectivity, which result from the hairpin stem-loop structure attained by the MB in the nonhybridized state (not associated to its target). The stability of the hairpin conformation is ensured by building more stable stems (either increasing its C-G content or the stem length). Unfortunately, increasing stem strength decreases also the hybridization rate. For a given MB the rates of hybridization decrease between one and two orders of magnitude when the stem length is increased from two to four nucleotides [27]. Shorter stems produce less stable hairpin structures, and in consequence reduce the signal background ratio and the selectivity of the MB-target interaction. For an optimal stem design, it is necessary to determine the balance between selectivity and hybridization rate for a given assay [26, 27, 68]. A typical MB has a stem of 5–7 nucleotides and a loop of 15–25 nucleotides in length (Figures 2(a) and 2(b)) and hybridize its target using only the loop region (Figure 2(b)). In order to accelerate the hybridization rate for MBs with more stable stems, one can increase the loop length, therefore augmenting the region of the MB that hybridize to the target. Another possibility, which prevents increasing the length of the loop, but ensures faster hybridization rates, is to include the stem sequence as part of the complementary region that will hybridize to the target. The MB could hybridize the target using one arm of the stem totally (Figure 2(c) for the 3’ arm or Figure 2(d) for the 5’ arm) or partially, or using partially both arms of the stem (Figure 2(e)). MBs using totally one arm of the stem to hybridize the target are known as shared stem (Figures 2(c) or 2(d)) [57]. For a given target sequence one shared-stem MB offers a high hybridization rate in comparison with a MB with equal number of complementary nucleotides, using only the loop as hybridization region, this is due to the complementary-nucleotides/MB-length ratio

\[
\text{Hybridization rate} \propto \frac{\text{complementary nucleotides}}{\text{MB-length}}.
\] (1)

For living cells assays the MB have to discriminate among perfect matched and single mismatched targets at 37°C, it means that the melting temperature of the MB-single-mismatched target pair have to be less than 37°C, while the melting temperature of the MB-perfect-matched target pair must be above 37°C [69]. This requirement could be covered during the design of the stem stability, loop length, and/or regions that will participate in the hybridization. If the MB is designed with too high MB-target melting temperatures, it will be impossible to differentiate between the perfect complementary and the single mismatch targets. On the other hand, it will be impossible to discriminate among perfect matched and single mismatched targets at 37°C.
hand, if the MB-target melting temperatures were reduced too much, just a little fraction of the perfectly complementary target would be bound to the MB at physiological temperatures.

4. Chemistry Approaches for High Performance Molecular Beacons

When a MB will be used for RNA imaging in living cells, it must have minimal requirements in terms of sensitivity and selectivity, to prevent false-positive signals derived from nonspecific interactions, like protein binding and/or nuclease digestion. One could imagine that if the MB were digested by a nuclease, then the separation of the quencher and the reporter would necessarily result in fluorescence signal, which would not reflect MB-target interactions. Similarly, if protein binding diminishes quencher efficiency, fluorescence increments will not reflect the expected MB-target association.

4.1. MB with Chemically Enhanced Sensitivity. Some RNA species have a very low number of copies in living cells (e.g., microRNAs), which represent a big challenge for detection using MB. In such case, it is necessary to build MB with enhanced sensitivity and increased signal to background ratio. Increasing the efficiency of the quenching or increasing the fluorescence intensity emitted by the reporter after target hybridization could accomplish this. Most of the recent strategies are focused on increasing the fluorescence intensity of the reporter.

A typical quenching efficiency by contact or static quenching is in the range of 85–97% [70], depending on the factors previously discussed. Increasing the quenching efficiency would necessarily result in increased signal to background ratios. The increment in the dynamic range increases the difference of the fluorescence emitted by a population of MB-perfect-matched target hybrids in comparison with an equivalent population of MB-single-mismatched targets.

Recently developed MB synthesis strategies to increase the quenching efficiency consist in adding several quencher molecules in tandem (the so-called superquenching). Recent MBs have been synthesized using a FAM reporter and two or three Dabcyl molecules as quenchers (Figure 3) at the 5′ end [19]. This strategy results in an increment in quenching efficiency from 92.9% for one Dabcyl to 98.75% for two Dabcyl and 99.7% for three Dabcyl quenchers. Also the signal enhancement increases from 14 (one Dabcyl) to 80 (two Dabcyl) and 320 units (three Dabcyl molecules), increasing significantly the chances of detecting very low quantities of target [19]. Another interesting observation resulting from the use of three Dabcyl in tandem is that the increase in the hydrophobic interactions that occurs between the reporter and quenchers cause that the melting temperature of the MB stem increases in 4°C in comparison with stems having a single Dabcyl quencher. In theory, the higher melting temperature could enhance the capability of the MB to discriminate between perfect matched and single mismatched targets.

Since fluorescence quenching is based on static and dynamic quenching mechanisms and the efficiency of both depends on molecular distances and spatial orientation (dipole moment), having multiple quenchers in tandem may facilitate that one or more of them attain the correct spatial position in respect to the reporter, thus explaining the high efficiency of the super-quenching phenomenon.

Some polymeric fluorescent dyes could be employed to increase the fluorescence intensity and also the sensitivity of MB. Poly(phenylene ethynylene) (PPE) [71] is a water soluble polyelectrolyte with a high quantum yield [72]. Using a novel polymerization reaction it is possible to couple PPE directly to oligonucleotides. PPE have the highest fluorescent intensity compared to common fluorescent dyes used in MB synthesis. PPE has a fluorescent intensity around 20-fold higher than Cy3 and more than 6-fold in comparison with Alexa Fluor 488 (the fluorescent dye with highest fluorescence intensity currently used for MB building). In addition the fluorescence intensity of a single PPE chain is about 75% of the brightness intensity obtained with a quantum dot. The Dabcyl quencher shows good quenching performance with PPE. Nevertheless, super-quenching with Dabcyl could increase the performance of a PPE-based MB and enhance the signal to a level that may facilitate the identification of low abundance RNA molecules in living cells.

4.2. Nuclease Resistant MB. Cells use nucleases for nucleic acid catabolism and reuse the nucleotides for nucleic acid synthesis. Nucleases are used for cellular defense against foreign nucleic acids and for degradation of damaged DNA. Unfortunately for the researcher, nucleases also deplete MB [73, 74], which constitutes a problem for RNA imaging in living cells using these probes. Recent experiments show that living cells exhibit increments in fluorescence intensity after 45 minutes of MB delivery, even in the absence of a target [75]. Data indicate that this fluorescence increment is due to MB nuclease-mediated degradation. To produce nuclease resistant MB with improved stability at the cytoplasm, modified nucleotides have been incorporated in MB synthesis. These modified nucleotides include 2′-O-methylated [76–78], phosphorothioate derivatives [79, 80], peptide nucleic acids (PNA) [81, 82], and locked nucleic acids (LNA) [83]. 2′-O-methylated MBs offer good nuclease resistance and also resist RNase activity [76]; the main disadvantage for its use is the high background due to nonspecific interactions with proteins [84–86], another common problem is probe accumulation at the nucleus [87] and mitochondria [88]. In order to avoid the nuclear accumulation a quantum dot (QD) has been recently linked to the 2′-O-methylated MB [89], the linker is a biotin-dT group at the 3′ end. The slow accumulation of the 2′-O-methylated MB at the mitochondria is reported only when using cyanine labeled MBs. PNA-based MBs have good affinity for both DNA and RNA targets in conjunction with high resistance to nuclease degradation. However, their reduced solubility and occasional aggregation limits its use for in vivo localization studies. LNA have a bicyclic furanose unit locked in an RNA-mimicking sugar conformation. LNA-based MBs show higher affinity for its target than DNA-based MBs; also the hybridization rate is slower in comparison. The use of chimeric DNA-LNA MBs results in nuclease resistant probes,
with very high selectivity. It is important to take in consideration the LNA/DNA ratio of the chimeric probes, and if LNA bases will be part of the stem or not. By lowering the DNA/LNA ratio the hybridization rate is increased but also the nuclease degradation [83]. If the LNA bases are located at the stem, MB stability increases significantly, improving the selectivity. A shared-stem design with a four basepair stem and alternating DNA/LNA bases (ratio 1:1) has been recently suggested for living cell applications [90]. With this design a reasonable hybridization rate is obtained in addition to lowering unspecific protein interactions and high nuclease resistance.

A novel strategy to avoid the possibility of the stem interactions with other nucleic acids or MB-MB between sticky ends consists in the use of L-DNA (specular isomer for the D-DNA) in the stem region [91]. D-DNA cannot interact with L-DNA and form left handed double helix. This design increases stem stability and MB selectivity.

### 5. Methods for MB Delivery into Cells

The efficient delivery of the MB into living cells is not an easy task. The method used must be efficient in terms that sufficient probe must be introduced in order to detect even low abundance RNA. Many delivery methods have been explored for MB introduction including microinjection, reversible cell membrane permeabilization, electroporation, MB linked to cell-penetrating peptides, and bioballistics. Needless to say that there is not a single method that can be used in all experimental conditions, therefore method selection is also based on the sample utilized.

Many transfection agents produce punctuate fluorescent patterns due to the passage of the MB by the endocytic pathway. Experimental evidence shows that linear fluorescently labeled probes enter in the endosomal/lysosomal pathway with the concomitant nuclease-mediated degradation. Reduced amounts of the probe may escape from the endocytic

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**Figure 3**: Structures of the superquenched MB. MB with one (1Q), two (2Q), and three (3Q) Dabcyl quenchers. The structures are represented in their oxidized state. The linker structures, the reporter linked to the 3' end and the quencher or quenchers attached at the 5' terminus correspond to [19].
pathway (0.01–10%) after several hours. For these reasons a delivery method in which the MB could enter the cell cytoplasm evading the endosomal pathway is highly desirable (e.g., microinjection, permeabilization of cell membranes by toxins, MB linked to cell penetration peptides, electroporation, or bioballistics).

5.1. Microinjection. The microinjection [42, 92] is in fact the most invasive method for MB delivery into living cells, which may result in cell damage and/or cell death. In the case where cells do not die, the injection could interfere with normal cellular functions and also produce altered or not reproducible results. Another disadvantage is the low number of cells that could be injected, given the fact that one must inject cell by cell. The major advantage is that low MB volumes are used reducing the cost of MB delivery. When using this method it is important to include a nonfluorescent dye (ink) in the solution, to ensure that the small volume enters the cell. Ink selection is also relevant to reduce side effects. Perhaps the main constrain for using this method is that a specialized injection system must be used in order to deliver a few nanoliters (nL) into the cell. Typically mammalian cells cannot be injected with volumes larger than 5 nL without compromising cell viability.

5.2. Toxic-Mediated Cell Membrane Permeabilization. A nonendocytic delivery method is the reversible membrane permeabilization mediated by toxins. Streptolysin O (SLO) is a bacterial toxin produced by streptococci hemolytic strain (Streptococcus pyogenes) having the ability to form pores on the cell membrane [93–95]. SLO binds as a monomer to cholesterol located on the cell plasma membrane, later SLO oligomerizes into a ring structure, producing a transient pore with a diameter in the range of 25–30 nm. The pore allows the entrance of macromolecules like the MB. The SLO cell membrane permeabilization method must be conducted in serum-free conditions; after membrane permeabilization the addition of the culture media containing serum inactivates the toxin [93, 96]. Since cholesterol composition is not a constant among cell types, the permeabilization process have to be optimized for every cell type, testing the MB entrance efficiency under different conditions of temperature, SLO concentration, cell number, and incubation time. We have found the use of combined MB-target solutions extremely useful for such testing to visualize the increase of cytosolic fluorescence as the MB-target enter the cell via the SLO pore.

5.3. MB Linked to Cell-Penetrating Peptides. There are several naturally occurring peptides with the capacity to penetrate cellular membranes (CPP: cell-penetrating peptides) [97–99]. One of the most widely used is, for instance, the transactivating transcriptional activator (Tat) from human immunodeficiency virus (HIV-1). Another peptide is a fragment of the Drosophila antennapedia homeodomain (RQIKIWFQNRRMKWKK) and the peptide VP22 from the tegument of the herpes simplex virus type 1 (HSV-1). In general, CPPs are short peptides rich in basic amino acids presenting an amphipathic arrange. CPPs penetrate across the cellular membranes without toxic effects and with very high efficiency (near to 100% of cells exposed are affected). The mechanism underlying CPP internalization prevents membrane destabilization, loss of cellular integrity and appears to be independent from the endocytic pathway (therefore reducing MB degradation by nuclease attack). Currently the most widely used CPP is the HIV-1 Tat peptide, and also the best studied due to its small size and high delivery efficiency.

CPPs deliver a wide variety of cargo molecules in culture cells or tissues [100, 101]. A major advantage of CPPs is that they do not interfere with specificity or sensitivity of MBs. Recently CCP and NLS peptides (peptides with nuclear localization sequences) were conjugated to MB by different linkers (being the most common the carbon saturated chains (Figures 5(b), 5(b’) and 5(c)). The maleimide-thiol system is a very useful tool to conjugate MB with peptides. Maleimide reacts with moieties having thiol groups (Figure 4(a)), like cysteine or free thiol groups. The first option is to have a sulphydrl in the terminus of the MB linker to react with maleimide at the terminus of the peptide (Figure 4(b)). The second option is the opposite, in that the MB linker has the maleimide and the peptide has the thiol group (Figure 4(b’)). A third option consists in having two sulphhydryl groups, one at the terminus of the MB linker and another at the terminus of the peptide (Figure 4(c)) forming a disulphide bond. This last option is the most versatile, because it facilitates the separation of the MB and the peptide using a reducing environment. Using CCPs as tools made possible imaging mRNAs like GAPDH recently [102]. Another interesting strategy involves the linking of the MB to peptides with nuclear localization signals (NLSs), in conjunction with SLO facilitates reaching nuclear targets like U1, U2, or U3 [103] (Figure 4(d)).

5.4. Electroporation. Electroporation methods have been used for delivering MBs into the cytoplasm of living cells, because electroporation avoid the endosomal pathway and in consequence reduces MB degradation. In the past, electroporation was associated with low cellular viability, but recent advances in this technology have led to a reduction in the events associated with cell damage or stress, including heat generation, metal ion dissolution, pH variations, or free radicals generation. Recently a novel electroporation method called microporation shows that a short time after the electroporation process occurs, a uniform distribution of the probe is observed at the cytoplasm [104]. Using this novel method high delivery efficiencies superior to 90% and cell viability near to 86% are observed [104]. With microporation (or any electroporation method) cells must be in suspension during the delivery procedure, therefore a time is required for cell to adhere before imaging studies can be conducted. This waiting period prevents the observation of early events of RNA mobilization or transport, a limitation to be taken into account during experiment planning.

5.5. Bioballistics. In the bioballistics technique, heavy metal particles are coated with molecules (e.g., MB) and propelled into the cells via a gas gun. The first application of this
**Figure 4:** MB linked to penetrating peptides. (a) Reaction of conjugation among the maleimide molecule with a carrier group (R₁) and the thiol group of another molecule. This reaction may possibly link MB-peptide and occurs at a pH between 6.5–7.5. (b) One possibility using the maleimide-thiol system is that the peptide (NLS or CCP) it is linked to the maleimide across the secondary amine and reacts with a sulfhydryl group at the terminus of the MB hydrocarbon linker. (b’) The other possibility for the maleimide-thiol system is that the maleimide is linked at the terminus of the MB hydrocarbon linker and reacts with a thiol group at the peptide (NLS or CCP). (c) The link between the hydrocarbon linker of the MB and the peptide also could be across a disulphide bridge, in this case exists the possibility to cleave the bond in a reducing environment. (d) Using SLO to make permeable the cytoplasmic membrane is possible to introduce a MB linked to an NLS peptide to the cytoplasm with the objective that it be transported into the nucleus by the cellular machinery. For a CCP-linked MB the entrance to the nucleus is impossible.
Figure 5: Approaches for RNA visualization in living cells. (a) In order to reduce the possibility of false-positive signals and also increase the specificity, one can design two shared stem MB having a pair reporters compatible with FRET, one to serve like a donor dye (red circle) and the other like a acceptor dye (green circle). With this strategy the sequence to be recognized increases its length and thus the specificity of the assay (observe that one MB needs to have the reporter at the 3’ end and the quencher at the 5’ end, in a not conventional attachment). After the MB design and synthesis the probe could be delivered using microinjection; in conjunction with other MB or fluorescent dyes could be used to measure RNA distribution at subcellular level. For example it is possible to use a specific fluorescent dye for the endoplasmic reticulum (ER) and also a MB designed to hybridize a given sequence of the ribosome. If a particular mRNA (based on the fluorescence of the MB) colocalizes with the ER dye and ribosome dye it means that this mRNA is translated in ribosomes at ER (b) or if the mRNA signal colocalizes only with the ribosome dye means that the mRNA translation occurs at free ribosomes (c); also it is possible that the mRNA translation takes place at both free and ER associated ribosomes. These are only some examples of the power of using MB in combination with other compartment or structure-specific dyes.

6. Real-Time Monitoring of RNA in Living Cells Using MB

6.1. mRNA Imaging in Living Cells Using MB. MBs have been only recently used for monitoring mRNA in living cells. MBs are particularly suited for mRNA studies because these RNA molecules are usually in high abundance, or at least higher than noncoding RNAs, for instance. For a successful MB design one needs to take into consideration all the factors involved in mRNA recognition by MB. Perhaps the most complicated issue is the selection of an appropriate target region in the mRNA sequence. One must first analyze the predicted secondary and tertiary structures in the mRNA target in an attempt to identify those regions showing minimum structure [68, 110]. In general, heavy secondary structure hampers MB binding to the target at room temperature, or results in very slow association kinetics. One should always consider that the determination of the target secondary structure is based on in silico predictions, which may or may not reflect the real structure of that region selected in vivo.
Another relevant consideration is to ensure that the region selected is not present in other nucleic acid in the cell. This can be partially accomplished using alignment tools like Blast [111], using the MB sequence as input. To ensure that the MB hybridizes with the expected target, a clever strategy has been recently implemented. In this strategy, the use of two shared stem MBs (one 3’ shared stem and the other 5’ shared stem) in adjacent positions is required (Figure 5(a)), both MBs are designed as FRET pair reporters to eliminate the possibility that false positives detection occurs [112, 113]. Shared stem design reduces the degree of freedom in the movements of both components of the FRET pair, enhancing FRET efficiency. Using two MBs reduces significantly the possibility of false positives, since having adjacent sequences in a given RNA that is recognized by two independent MBs is equivalent to recognizing a larger sequence (the sum of the MB-target complementary nucleotides in every MB).

Another promising application for MB research involves its use in conjunction with fluorescent dyes for specific organelles or subcellular structures. For example an assay designed to identify if a given mRNA is translated at ribosomes associated with endoplasmic reticulum (ER) or in free ribosomes could be developed, using a selective fluorescent dye for the ER and a MB designed to hybridize with the ribosomes and a second MB designed to detect the mRNA. With these elements it is possible to observe the colocalization of the mRNA within the ER and ribosomes simultaneously (Figure 5(b)) or visualize the colocalization of the mRNA with ribosomes only (no ER labeling) if the translation occurs at free ribosomes (Figure 5(c)).

### 6.2. Monitoring Gene Expression in Living Cells

The first assays where MB were involved focused only in detecting synthetic targets in in vitro studies, but in 2003 Bratu et al. demonstrated that a MB constitutes a powerful tool for RNA imaging and could be used for visualizing the distribution and transport of mRNA in living cells [48]. To reduce the background noise and ensure that the MB hybridizes its target, two MBs that recognize adjacent positions at the target were designed. The binding of both MBs resulted in FRET signal (energy transfer from one MB to the adjacent MB), indicating that both MBs were bound to adjacent regions in the target [112, 113]. The robustness of this method allows not only the identification of the mRNA target localization in the cell, but also the possibility to follow the transport of the mRNA through the cell in real time. Diffusion coefficients of the mRNA can be accurately measured combining fluorescence recovery after photobleaching (FRAP). In addition Santangelo et al. were beyond and analyzed the distribution and transport of the mRNA with respect in organelles from human fibroblasts. For this study MBs were used in combination with fluorescent stains of mitochondria, using spectral resolution Santangelo et al. demonstrated that both mRNA for glyceraldehyde 3-phosphate dehydrogenase and K-ras were specially enriched within the mitochondria [112].

Recently Wang et al. described a method to determine the expression levels of a specific RNA using MBs [114]. They use a system with two MBs and a reference probe as an internal standard for ratiometric analysis. The reference probe was used to avoid the variations due to different MB concentrations in the cell. One MB was used to detect the desired mRNA whereas the other MB was used to hybridize a house keeping mRNA, serving as reference. Three different cell types were used to compare expression levels of the mRNA of interest with excellent results.

Even though several methods for the study of unique mRNA molecules have been implemented, monitoring multiple RNA molecules in the same cell remains a technical challenge. The ratiometric analysis offers a good approach for the quantification of gene expression [115], enabling a more accurate estimation of mRNA levels. Even though in this particular study two different MBs were used, one can envision an extension of this method to study several RNA molecules simultaneously using spectrally different fluorescent reporters for each MB.

### 6.3. Monitoring Viral Genomic RNA Replication in Living Cells

RNA viruses have a genome composed by RNA with structural differences, which allow their classification into groups. Three groups of RNA viruses have been described, group III with a segmented double-stranded RNA genome, group IV with positive-sense single-stranded RNA genome, and group V with a negative-sense single-stranded genome. Methods for live-cell imaging of viral genomic RNA replication using MBs are currently available. Recently a method was implemented to monitor virus replication in living cells with the B6 coxsackievirus [116]. Virus replication was monitored using 2’-O-methylated RNA-MB. MB was introduced in the cells using a thiol-maleimide bridge linked to the Tat peptide. The MB was directed to a sequence of 18 basepairs located in the 5’ noncoding region of the viral genome. Coxsackieviruses belong to a Picornaviridae family having a positive-sense single-stranded genome. In a different report, researchers monitored genomic RNA replication of the bovine respiratory syncytial virus (bRSV) [46] using a MB directed to tandem-repeated consensus-sequences at intergenic regions, in order to amplify the signal. Detection of viral genomic RNA in living cells is a promising application because it facilitates the detection of viruses without the need for RNA purification, cDNA synthesis steps and/or amplification procedures, and also open the possibility of obtaining spatial-temporal information contributing to the understanding of viral replication, delivery, or propagation mechanisms. These contributions could facilitate the development of drugs for viral infection treatment or rapid diagnostics techniques. Undoubtedly, MBs will impulse viral research by facilitating the study of genome replication and transport in living cells, something difficult to envision before the MB era.

### 7. Conclusion

We have discussed in this paper important advances in the design, synthesis, and use of MBs since its introduction by Tyagi and Kramer in 1996. We have highlighted relevant issues involved in sequence selection, loop, and stem considerations needed for a successful MB design. We
have mentioned also new developments in MB synthesis using modified nucleic acids highlighting its advantages and limitations. Finally, we have gathered recent studies where MBs have been used in rather clever experiments directed to the study of RNA localization and dynamics in living cells.

The development of novel fluorescent indicators with greater dynamic range in combination with robust quenchers, in the next generation of MBs, will provide exceptional tools for RNA studies in living cells and tissues. The combination of such tools with powerful imaging techniques, such as FRAP, FLIM, TIRFM, and multiphoton confocal microscopy promises the possibility to conduct such studies in living organisms.

Finally, a poorly explored area in MB research is the use of these tools in diagnostics. One can easily envision methodologies capable of identifying replicating viruses in human samples, using MB in combination with fluorescent assays without the need of time consuming more elaborated techniques such as qPCR.

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References


Review Article

Resveratrol, MicroRNAs, Inflammation, and Cancer

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MicroRNAs are short noncoding RNAs that regulate the expression of many target genes posttranscriptionally and are thus implicated in a wide array of cellular and developmental processes. The expression of miR-155 or miR-21 is upregulated during the course of the inflammatory response, but these microRNAs are also considered oncogenes due to their upregulation of expression in several types of tumors. Furthermore, it is now well established that inflammation is associated with the induction or the aggravation of nearly 25% of cancers. Therefore, the above microRNAs are thought to link inflammation and cancer. Recently, resveratrol (trans-3,4′,5-trihydroxystilbene), a natural polyphenol with antioxidant, anti-inflammatory, and anticancer properties, currently at the stage of preclinical studies for human cancer prevention, has been shown to induce the expression of miR-663, a tumor-suppressor and anti-inflammatory microRNA, while downregulating miR-155 and miR-21. In this paper we will discuss how the use of resveratrol in therapeutics may benefit from the preanalyses on the status of expression of miR-155 or miR-21 as well as of TGFβ1. In addition, we will discuss how resveratrol activity might possibly be enhanced by simultaneously manipulating the levels of its key target microRNAs, such as miR-663.

1. Inflammation and Cancer

Inflammation represents a complex, nonspecific immune response of the body to pathogens, damaged cells, tissue injury, allergens, toxic compounds, or irritant molecules [1]. While it is normally self-contained, it may become permanent and chronic. It may also escape the original tissue and spread via the circulatory and/or the lymphatic system to other parts of the body, producing a systemic inflammatory response syndrome such as sepsis in case of an infection. Chronic inflammation is associated with a simultaneous destruction and healing of the tissue from the inflammatory process and has been linked to a number of pathologies, including cancer, chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases, and psoriasis, as well as several types of neurological disorders. The inflammatory response is coordinated by a large range of mediators that form complex regulatory networks [1]. The recruitment of leukocytes in the peripheral tissues is the hallmark of inflammation. It is mediated by several types of chemokines [1–3], which act through their receptors located at the surface of the cytoplasmic membrane of leukocytes. The production of chemokines is induced by inflammatory stimuli such as bacterial lipopolysaccharide (LPS), interleukin (IL)-1, or tumor necrosis factor (TNF). In addition, these chemokines have a clear role in angiogenesis and wound repair [4]. Finely tuned molecular mechanisms exist that ensure that the immune response required for the defense of the body has a limited duration and does not pass a certain maximum activation level, thus avoiding that, it becomes harmful to the organism. It is well acknowledged that unresolved immune response results in inflammation.

Epidemiological studies suggest that as many as 25% of all cancers may be due to chronic inflammation [5–7]. The connection between inflammation and cancer consists of
an extrinsic pathway, driven by inflammatory conditions that increase cancer risk, and an intrinsic pathway, driven by genetic alterations that cause inflammation and neoplasia [6]. Inflammatory mediators released by cancer-related inflammation induce genetic instability, leading to the accumulation of random genetic alterations in cancer cells [7]. The activation of Toll-like receptors (TLRs), a group of pattern recognition receptors functioning as sensors of pathogens and tissue damages, leads to the nuclear translocation of NF-κB and the production of cytokines such as TNF, IL-1α, IL-1β, IL-6, and IL-8. However, TLR activation has been shown to accelerate the growth of adoptively transferred tumors [8–11]. Accordingly, the stimulation of TLRs leads to increased survival and proliferation of several cell lines [12, 13], and the intratumoral injection of *Listeria monocytogenes* induces TLR2 signaling in tumor cells, thus promoting their growth [14]. TLR signaling also enhances tumor cell invasion and metastasis by regulating metalloproteinasises and integrins [15]. Chemokines also affect several tumor progression pathways, such as leukocyte recruitment and function, cellular proliferation, survival, or senescence, as well as invasion and metastasis and are the targets of a number of anticancer agents [5].

Tumor microenvironment contains various inflammatory cell types infiltrating the tumor area in response to inflammatory stimuli, such as macrophages, neutrophils, and mast cells [16, 17]. Tumor-associated macrophages (TAMs) are thought to play key roles in the production of various growth factors, angiogenic factors, proteinases, chemokines, and cytokines, through crosstalks with cancer cells and other tumor stromal cells [18–20]. Factors secreted by TAMs stimulate cell migration/motility, proliferation, survival, angiogenesis, and metastasis, resulting in a dynamic environment that favors the progression of cancer, thus affecting the clinical outcome of malignant tumors. TAMs have thus been described as “obligate partners for tumor-cell migration, invasion and metastasis” [21, 22]. Namely, in a genetic model of breast cancer in macrophage-deficient mice, the tumors developed normally but were unable to form pulmonary metastases in the absence of macrophages [23]. As tumor metastasis is responsible for approximately 90% of all cancer-related deaths, a better understanding of inflammation regulatory mechanisms may potentially allow to optimize the use of anticancer drugs that lower tumor-specific inflammatory response [18].

Finally, the transforming growth factor β (TGFβ) regulates the immune response as well as the effects of the immune system on tumor progression or regression in vivo [24]. TGFβ has been shown to suppress the antitumor activity of T cells, natural killer (NK) cells, neutrophils, monocytes, and macrophages, which together are able to promote or repress tumor progression depending on the cellular context [25–27]. Importantly, TGFβ1, the most abundant and ubiquitously expressed isoform of TGFβ, is usually considered a tumor-suppressor, due to its cytostatic activity in epithelia. However, on advanced stages of tumors, TGFβ1 behaves as a tumor promoter, due to its capability to enhance angiogenesis, epithelial-to-mesenchymal transition, cell motility, and metastasis [28–30].

### 2. MicroRNAs and Inflammation

MicroRNAs (miRNAs) are short noncoding RNAs which regulate the translation and/or degradation of target messenger RNAs [31–33]. They have been implicated in the regulation of a number of fundamental processes, including muscle, cardiac, neural, and lymphocyte development, or the regulation of both the innate and adaptive immune responses [34, 35]. miRNAs originate from primary transcripts (pri-miRNAs) converted in the nucleus into precursor miRNAs (pre-miRNAs) by the RNase III Drosha, associated with DGC8R to form the small microprocessor complex [36]. Pre-miRNAs are then exported in the cytoplasm where the miRNA hairpin is cleaved by the RNase III Dicer within the RISC loading complex. The guide strand, which corresponds to the mature miRNA, is then incorporated into the RISC complex [36]. miRNAs and their transcriptional regulators usually form autoregulatory loops aimed at controlling their respective levels [37]. miRNAs participate in many gene regulatory networks whose molecular malfunctions are associated with major pathologies such as cancer [34] or auto-immune diseases [38–40].

Several miRNAs have been implicated in both inflammation and cancer [38–41]. The most prominent are miR-155, miR-21, and miR-125b. Thus the expression of miR-155 is strongly elevated in several human leukemias and lymphomas ([40] and references therein). Transgenic mice with B cells overexpressing miR-155 develop B-cell leukemia, and a sustained expression of miR-155 in hematopoietic stem cells causes a myeloproliferative disorder [40]. On the other side, miR-155-5p has been implicated in the regulation of myelopoesis and erythropoesis, Th1 differentiation, B-cell maturation, IgG1 production, somatic hypermutations, gene conversion, class switch recombination, and B- and T-cell homeostasis as well as in the regulation of the innate immune response [40]. Thus, miR-155 levels increase following LPS treatment of Raw-264 macrophages, and miR-155 transgenic mice show enhanced sensitivity to LPS-induced endotoxin shock [42]. In contrast, miR-155-knock-out mice are unable to mount a proper T-cell or B-cell immune response [40]. The expression of another miRNA, miR-125b, was repressed within 1 hour of LPS challenge in Raw-264 cells [42]. In topic eczema, miR-125b expression was reduced in regions of the skin that were inflamed, while that of miR-21 was enhanced [43]. Similarly, miR-21 expression was increased by inflammation due to ulcerative colitis [44] and also by IL-13 and by specific antigens in OVA- and *Aspergillus fumigatus* antigens-induced asthma models [45]. The expression of miR-21 changes dynamically during antigen-induced T-cell differentiation, with the highest levels of expression in the effector T cell [46]. MiR-21 induction upon T-cell receptor (TCR) stimulation is believed to be involved in a negative feedback loop regulating TCR signaling [46]. MiR-663 has drawn recent attention due to its role not only as an anti-inflammatory miRNA but also as a tumor suppressor miRNA. Thus, MiR-663 impairs the upregulation of miR-155 by inflammatory stimuli [47]. In addition, the expression of this microRNA is lost in certain cancers such as gastric or pancreatic cancer, and it induces mitotic catastrophe...
growth arrest when its expression is restored in these cells [48].

3. MicroRNAs as Oncogenes and Tumor-Suppressor Genes

miRNAs participate in many gene regulatory networks whose molecular malfunctions are associated with cancers [35, 41]. Depending on the effects of their downregulation or overexpression, miRNAs have been described either as onco-genic (onco-miRs) or tumor suppressors. Thus, the miR-17-92 cluster on chromosome 13, which contains six miRNAs (miR-17, -18a, -19a, -20a, -19b-1, and -92a-1), is amplified and overexpressed in B-cell lymphomas and solid tumors such as breast or small-cell lung cancers, where it may enhance oncogenesis by potentially targeting E2F1, p21/CDKN1A, and BCL2L11/BIM [49]. On the other hand, it may enhance oncogenesis by potentially targeting E2F1, p21/CDKN1A, and BCL2L11/BIM [49]. On the other hand, it may enhance oncogenesis by potentially targeting E2F1, p21/CDKN1A, and BCL2L11/BIM [49]. On the other hand, it may enhance oncogenesis by potentially targeting E2F1, p21/CDKN1A, and BCL2L11/BIM [49]. On the other hand, it may enhance oncogenesis by potentially targeting E2F1, p21/CDKN1A, and BCL2L11/BIM [49]. On the other hand, it may enhance oncogenesis by potentially targeting E2F1, p21/CDKN1A, and BCL2L11/BIM [49]. On the other hand, it may enhance oncogenesis by potentially targeting E2F1, p21/CDKN1A, and BCL2L11/BIM [49].

In human, the levels of both miR-155 and BIC transcripts (that is, miR-155 primary RNAs) are elevated in diffuse large B-cell lymphoma (DLBCL), Hodgkins lymphoma, and primary mediastinal B-cell lymphoma [40]. In contrast, a very weak expression of miR-155 is found in most non-Hodgkins lymphoma subtypes, including Burkitt lymphoma. In addition, high levels of BIC and miR-155 expression were reported in B-cell chronic lymphocytic leukemia (B-CLL) and in B-CLL proliferation centers [56]. Furthermore, miR-155 was also upregulated in a subset of patients with acute myelomonocytic leukemia and acute monocytic leukemia. Accordingly, transgenic mice whose B cells overexpress miR-155 developed polyclonal preleukemic pre-B-cell proliferation followed by B-cell malignancy [40]. On the other hand, it was reported that BIC cooperates with c-Myc in avian lymphomagenesis and erythroleukemogenesis [57]. Beside liquid malignancies, high levels of miR-155 expression were found in solid tumors such as breast, colon, and lung cancers [40]. miR-155 was recently shown to induce a mutator phenotype by targeting Wee-1, a kinase regulating G2/M phase transition during the cell cycle [58]. Furthermore, it was shown that miR-155 increases genomic instability by targeting transcripts encoding components of the DNA mismatch repair machinery [59]. As a consequence, the simultaneous miR-155-steered suppression of a number of tumor suppressor genes combined with a mutator phenotype might allow the shortening of steps required for tumorgenesis, and might also explain how chronic inflammation associated with high levels of miR-155 induces cancer.

Finally, miRNAs have been implicated in metastasis. For example, it has been established that the downregulation of both miR-103-1 and miR-103-2 miRNAs induces epithelial-to-mesenchymal transition by targeting Dicer1 transcripts [60]. Furthermore, several miRNAs, including miR-21, have been shown to activate metastasis by acting on multiple signaling pathways and targeting various proteins that are involved in this process. Thus, in breast cancer, which represents the most common malignancy among women in the world, miRNAs such as miR-9, miR-10b, miR-21, miR-103/107, miR-132, miR-373, and miR-520 stimulate metastasis, while miR-7, miR-30, miR-31, miR-126, miR-145, miR-146, miR-200, miR-205, miR-335, miR-661, and miRNAs of the let-7 families in contrast impair the different steps of metastatic process, from epithelial-to-mesenchymal transition to local invasion to colonisation and angiogenesis [61].

Numerous reports have provided strong evidence that all of the above miRNAs potentially target a myriad of transcripts including those encoding transcription factors, cytokines, enzymes and kinases, implicated in both cancer and inflammation.

4. Anti-Inflammatory and Antitumor Properties of Resveratrol

Resveratrol (trans-3,4’,5-trihydroxystilbene) is a natural polyphenolic, nonflavonoid antioxidant found in grapes and other berries, produced by plants in response to infection by the pathogen Botrytis cinerea [62]. Recent studies have documented that resveratrol has various health benefits, such as cardiovascular- and cancer-preventive properties [63–65], and this compound is currently at the stage of preclinical studies for human cancer prevention [66, 67]. Resveratrol was first shown to inhibit both tumor promotion and tumor progression in a mouse skin cancer model [68]. Resveratrol is also tested for preventing and/or treating obesity and diabetes [69, 70]. Fortunately, resveratrol toxicity is minimal, and even proliferating tissues such as bone marrow or intestinal tract are not adversely affected [71].

Resveratrol exerts its effects at multiple levels. Both its m-hydroxyquinone and 4-hydroxystyryl moieties have been shown to be important for the determination of resveratrol inhibitory properties toward various enzymes. This include lipoxigenases and cyclooxygenases that synthesize proinflammatory mediators from arachidonic acid, protein kinases such as PKCs and PKD, and receptor tyrosine kinases, lipid kinases, as well as IKKα, an activator of NF-κB pathway, which establishes a strong link between inflammation
and tumorigenesis [72]. Also, resveratrol inhibition of P450/CYP19A1/Aromatase, by limiting the amount of available estrogens and consequently the activity of estrogen receptors, has been proposed to contribute to the protection against several types of cancer, including breast cancer [72, 73]. Of note, resveratrol also inhibits the formation of estrogen-DNA adducts, which are elevated in women at high risk for breast cancer [74].

Resveratrol in addition regulates apoptosis and cell proliferation. It induces growth arrest followed by apoptotic cell death and interferes with cell survival by upregulating the expression of proapoptotic genes while simultaneously downregulating the expression of antiapoptotic genes [75]. Resveratrol induces the redistribution of CD95 and other death receptors in lipid rafts, thus contributing to their sensitization to death receptor agonists [75]. It also causes growth arrest at G1 and G1/S phases of cell cycle by inducing the expression of CDK inhibitors p21/CDKN1A and p27/CDKN1B [63]. In addition, resveratrol directly inhibits DNA synthesis by diminishing ribonucleotide reductase and DNA polymerase [72, 76, 77]. Altogether, antiproliferative activities of resveratrol involve the differential regulation of multiple cell-cycle targets in a cell-type-dependent manner [72, 75].

One of the possible mechanisms for resveratrol protective activities is by downregulation of the inflammatory responses [78]. That includes the inhibition of synthesis and release of proinflammatory mediators, modifications of eicosanoid synthesis, or inhibiting the enzymes, such as cyclooxygenase-1 (COX-1/PTGS1) or -2 (COX-2/PTGS2), which are responsible for the synthesis of proinflammatory mediators, through the inhibitory effect of resveratrol on transcription factors like NF-κB or activator protein-1 (AP-1) [78, 79]. Of note, constitutive COX-2 expression generally predicts aggressiveness of tumors, therefore, the use of nonsteroidal anti-inflammatory drugs that inhibit COX-2 in cancer treatment. However, cytoplasmic COX-2 can relocalize in the nucleus. This nuclear relocalization of COX-2 is induced by resveratrol, and exposure of resveratrol-treated cells to a specific COX-2 inhibitor blocked resveratrol-induced apoptosis, indicating that COX-2 displays proapoptotic activity in the nucleus, which may be associated with the generation of complexes of COX-2 and ERK1/2 mitogen-activated protein kinases. In mouse macrophages, resveratrol also displays antioxidant activity, decreasing the production of reactive oxygen species and reactive nitrogen species and also displays antioxidant activity, decreasing the production of reactive oxygen species and reactive nitrogen species and may be formed, the capability of resveratrol to specifically target a subset of AP-1 dimers through the upregulation of miR-663 might have profound effects on the levels of

5. **MiR-663 as a Mediator of Resveratrol Anti-Inflammatory Activity**

Affymetrix microarrays and RNase-protection assays showed that resveratrol treatment of human THP-1 mononuclear cells upregulated the expression of LOC284801 transcripts, that contain the sequence of pre-miR-663 and thus represent miR-663 primary transcripts. MiRNA microarrays and RNase-protection assays accordingly confirmed these data [47]. Interestingly, in silico analysis using TargetScan (http://www.targetscan.org/) suggested that miR-663 may potentially target transcripts encoding factors implicated in (i) the mounting of the immune response, especially JunB, JunD, and FosB, which encode AP-1 factors known to activate cytokine genes in partnership with NFAT factors [84], (ii) TLR signaling, such as the kinases RIPK1 and IRAK2, and (iii) the differentiation of monocytes, Th1 lymphocytes, and granulocytes.

An antisense miR-663 inhibitory RNA (663-1) proved capable of increasing global AP-1 activity in unchallenged THP-1 cells, showing that miR-663 indeed target transcripts encoding AP-1 factors in these cells. These effects were in particular directed toward JunB and JunD transcripts [47]. In agreement with previous results [79], resveratrol blocked the surge of AP-1 activity that occurs following LPS challenge due to the fact that JunB transcripts peak within the first hour, leading to the accumulation of JunB in the next few hours [85]. This inhibitory effect of resveratrol on AP-1 activity was partly impaired by 663-1, indicating that it arises at least in part from the upregulation of miR-663 by resveratrol [47]. Western blots showed that resveratrol impaired JunB neosynthesis, while still allowing the phosphorylation, that is, the activation of JunB following LPS treatment to take place, at least to a certain extent. Given that AP-1 factors include c-Jun, JunB, JunD, FosB, Fra-1, and Fra-2, as well as Jun dimerization partners JDP1 and JDP2 or the closely related ATF2, LRF1/ATF3, and B-ATF, so that potentially about 18 different dimeric combinations may be formed, the capability of resveratrol to specifically target a subset of AP-1 dimers through the upregulation of miR-663 might have profound effects on the levels of
the transcriptional activity of promoters to whom different AP-1 factors can compete for binding. Due to the many roles of AP-1 factors both in inflammation and cancer [86, 87], the specific targeting of genes encoding a subset of AP-1 factors, by changing the composition of AP-1 dimers on key promoters, may possibly explain some of the multiple anti-inflammatory and anticancer properties of resveratrol.

Of note, mir-155 had been shown to be under AP-1 activity in activated B cells [88]. Accordingly, mir-663 reduced the upregulation of mir-155 by LPS [42], which may be due to mir-663 targeting of transcripts encoding JunB and JunD and also possibly FosB and KSRP, an RNA binding protein implicated in the LPS-induced mir-155 maturation from its primary transcripts BIC [89]. This is of primary importance, for mir-155 upregulation is the hallmark of inflammatory response following LPS treatment of macrophages/monocytes [42]. Resveratrol also dramatically impaired the upregulation of mir-155 by LPS, an effect partly inhibited by 663-I [47]. Altogether, these results indicate that the anti-inflammatory properties of resveratrol arise, at least in part, from its upregulation of mir-663 and its downregulating effects on mir-155 and that mir-663 might possibly qualify as an anti-inflammatory miRNA.

6. MicroRNAs as Mediators of Resveratrol Anticancer Effects

The results reported here above also suggested that, due to its targeting of AP-1 factors, known to play a role in tumorogenesis and cell invasion [86, 87] and due to its downregulation of mir-155, whose levels increase in solid as well as in liquid tumors [40], mir-663 may also possibly provide resveratrol with some of its anticancer properties. Namely, mir-663 was found to be downregulated in hormone refractory prostate cancer cells, along with mir-146a and mir-146b [90], further supporting the hypothesis that this miRNA is a tumor-suppressor gene whose one of the function is to keep low the expression level of oncogenic mir-155 [47, 48].

CRC is the third most common malignancy and the fourth biggest cause of cancer mortality worldwide [91, 92]. Despite the increased use of screening strategies such as fecal occult blood testing, sigmoidoscopy, and colonoscopy, more than one-third of patients with colorectal cancer will ultimately develop metastatic disease [92]. On the other hand, the TGFβ signaling pathway is one of the most commonly altered cellular signaling pathways in human cancers [93]. Among the three TGFβ isoforms expressed in mammalian epithelia (TGFβ1, TGFβ2, and TGFβ3), TGFβ1 is the most abundant and ubiquitously expressed one. TGFβ signaling is initiated by the binding of TGFβ ligands to type II receptors (TGFβR2). Once bound by TGFβ, TGFβR2 recruits, phosphorylates, and thus activates the type I TGFβ receptor (TGFβR1). TGFβR1 then phosphorylates two transcriptional regulators, namely, SMAD2 and SMAD3, which subsequently bind to SMAD4. This results in the nuclear translocation of SMAD complexes, allowing SMADs to interact with transcription factors controlling the expression of a multitude of TGFβ responsive genes [94]. The expression of TGFβ1 in both tumor and plasma was found to be significantly higher in patients with metastatic colorectal cancer, and increasing colorectal tumor stage was correlated with higher TGFβ1 expression in tumor tissues [95].

miRNA microarrays recently showed that resveratrol treatment of SW480 human colon cancer cells significantly increased the levels of 22 miRNAs while decreasing those of 26 others [83]. Among the miRNAs downregulated by resveratrol, mir-17, mir-21, mir-25, mir-92a-2, mir-103-1 and mir-103-2 have been shown to behave as onco-miRNAs, at least in certain contexts. Thus, genomic amplification and overexpression of mir-17-92 miRNAs is found in B-cell lymphomas as well as in breast and lung cancers [34, 54]. Mir-21 is overexpressed in several cancers, including CRCs, gliomas, as well as breast, gastric, prostate, pancreas, lung, thyroid, and cervical cancers [53–55]. Mir-17, mir-25, mir-26-a, and mir-181a are also overexpressed in CRCs [34, 54]. In addition, several miRNAs, including mir-21, have been shown to activate metastasis by acting on multiple signaling pathways and targeting various proteins that are key players in this process [53]. Furthermore, the lower metastatic propensity of SW480 cells as compared with SW620 human colon cancer cells, both derived from the primary tumor and a metastasis of the same patient, respectively [96], was associated with a lower level of expression of mir-103-1 and mir-103-2, two miRNAs that induce epithelial-to-mesenchymal transition by targeting Dicer1 transcripts [60].

In silico analysis using TargetScan showed that miRNAs downregulated by resveratrol in SW480 cells potentially target transcripts encoding known tumor suppressor factors, such as the two antiproliferation factors PDCD4 and PTEN, the components of the mismatch repair machinery MLH3, MSH2 and MSH3, DICER1, the RNase III producing mature miRNAs from their immediate precursors in the cytoplasm, and several effectors and regulators of the TGFβ signaling pathway [83]. Indeed, resveratrol treatment of SW480 cells lead to a greater accumulation of TGFβ1R1, TGFβ2R, PDCD4, PTEN, and E-CADHERIN (a component of adherens junctions implicated in the maintenance of epithelial phenotype) [83]. Of note, among miRNAs upregulated by resveratrol, mir-663 was the only one to target TGFβ1 transcripts. Luciferase assays and Western blots showed that resveratrol downregulated TGFβ1 in both a mir-663-dependent and a mir-663-independent manner [83]. Resveratrol treatment also decreased the transcriptional activity of SMADs under TGFβ1 signaling, an effect seemingly independent of mir-663 [83].

Interestingly, it has been recently shown that GAM/ZNF512B, a vertebrate-specific developmental regulator first described in chicken [97], impairs the upregulation of miRNAs of the mir-17-92 cluster by TGFβ1 and that TGFβ1 in turn downregulates GAM, at least in part through the upregulation of mir-17-92 miRNAs [98]. The facts that GAM transcripts contain three consensus target sites for mir-663 and that GAM is sensible to resveratrol treatment (Tili et al., unpublished results) raises the question of the possible existence of a gene regulatory network that would allow mir-663 to impair GAM repressing activity on TGFβ1 signaling.
pathway when TGFβ1 works as a tumor suppressor, that is, at the early stages of tumorigenesis but not any more when this pathway starts to favor tumorigenesis and metastasis, that is, on advanced stages of cancers.

It is important to emphasize that TGFβ1 has been shown to enhance the maturation of oncogenic miR-21 through the binding of SMAD3 to miR-21 primary RNAs [99] and also to increase the expression of miR-155, which is under the control of TGFβ/SMAD activity [100]. Thus, by targeting AP-1 factors as well as TGFβ1 and possibly SMAD3, miR-663 might inhibit two of the pathways that upregulate miR-155 expression.

The TGFβ signaling pathway present multiple levels of regulation: sequestration of ligands into inactive precursor forms, ligand traps, decoy receptors, and inhibitory SMADs, not to mention the existence of SMAD-independent pathways and their interactions with many other critical signaling pathways which also proved to play a role in cancer. It is thus not surprising that the short (307-nt long) 3′-UTR of TGFβ1 transcripts contains a potential consensus target site for 28 miRNAs only. Of note, TGFβ1 3′-UTR contains two target sites for two of these miRNAs and only one target site for 25 of the others. Therefore, the fact that miR-663 may potentially target 5 different sites in TGFβ1 3′-UTR suggests that this miRNA could represent a critical TGFβ1 regulator which may possibly be called upon action in emergency situations such as when cells begin to proliferate anarchically or when a stronger immune response is required. The multiplicity of miR-663 targets sites in TGFβ1 3′-UTR further suggests that the effects of this miRNA might be both dose- and context-dependent, so that resveratrol effects on TGFβ1 signaling pathway might well be also context-dependent. Finally, it is probable that, depending on the cell context, resveratrol might either increase the level of TGFβ signaling—by inhibiting miRNAs targeting its main effectors—when it is beneficial to the organism, that is, when it works to maintain the integrity of epithelia and impair cell proliferation, or in contrast decrease TGFβ1 signaling—by decreasing its production through the upregulation of miR-663—when TGFβ1 starts to favor epithelial-to-mesenchymal transition and metastasis. For example, the targeting of both TGFβ1 and SMAD3 transcripts might possibly allow resveratrol to impair TGFβ1-induced SMAD3-dependent promotion of cell motility and invasiveness in advanced stages of gastric cancer [101, 102] or when SMAD2 and SMAD3 phosphorylated at both linker and COOH-terminal regions transmit malignant TGFβ1 signal in later stages of human CRC [103].

7. Conclusions

It is notable that striking phenotypes are often driven through small changes in the cellular concentration of key factors. For example, in the B-cell compartment, miR-150 curtails the activity of c-Myb transcription factor in a dose-dependent fashion over a narrow range of miRNA and c-Myb concentrations [104]. Thus, even slight effects of resveratrol on a handful of key miRNAs might well prove to be critical to its anti-inflammatory, anticancer and antimetastatic properties. In addition, the fact that miR-663, miR-21, miR-155, and TGFβ1 have all been implicated in the regulation of cell proliferation, tumor apparition and development, metastasis formation, and innate immunity, strongly suggests that the capability of resveratrol to behave at the same time as an antitumor, antimetastatic, antiproliferation and anti-inflammatory agent most probably arises from its effects on the expression of a small set of critical endogenous miRNAs having the abilities to impact the cell proteome globally.

Finally, miRNAs have the promise to become biomarkers for different stages of cancer, both for diagnosis and prognosis. Furthermore, the discovery that resveratrol can modulate the levels of miRNAs targeting proinflammatory and/or protumor factors opens the possibility to optimize resveratrol treatments by manipulating in parallel the levels of expression of a few critical miRNAs. For example, from the experiments reported here above, it starts to become clear that the use of resveratrol would be especially beneficiary in the type of cancers where the TGFβ pathway is implicated. Of course, resveratrol use would have to be carefully correlated with the stages of cancers, knowing that TGFβ can have two faces, that is, anti- and prometastatic.

As a last remark, it should be noted that while resveratrol antitumor potential has been linked with data primarily from human cell culture systems, evidence that resveratrol can inhibit carcinogenesis in several organ sites emerged from results of cancer prevention and therapy studies in laboratory animal models [68]. Given that miR-663 was only found in primates, the reports by Tili et al. [47, 83] come as a warning that studies in animal may not always allow to predict accurately the molecular effects of resveratrol in human, especially when it comes to miRNAs.

References


Research Article

Synthetic Pre-miRNA-Based shRNA as Potent RNAi Triggers

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RNA interference (RNAi) is a powerful tool for studying gene function owing to the ease with which it can selectively silence genes of interest, and it has also attracted attention because of its potential for therapeutic applications. Chemically synthesized small interfering RNAs (siRNAs) and DNA vector-based short hairpin RNAs (shRNAs) are now widely used as RNAi triggers. In contrast to expressed shRNAs, the use of synthetic shRNAs is limited. Here we designed shRNAs modeled on a precursor microRNA (pre-miRNA) and evaluated their biological activity. We demonstrated that chemically synthetic pre-miRNA-based shRNAs have more potent RNAi activity than their corresponding siRNAs and found that their antisense strands are more efficiently incorporated into the RNA-induced silencing complex. Although greater off-target effects and interferon responses were induced by shRNAs than by their corresponding siRNAs, these effects could be overcome by simply using a lower concentration or by optimizing and chemically modifying shRNAs similar to synthetic siRNAs. These are challenges for the future.

1. Introduction

RNA interference (RNAi) is an evolutionarily conserved, gene-silencing mechanism that is triggered by double-stranded RNA (dsRNA). Two types of small RNA—namely, small interfering RNA (siRNA) and microRNA (miRNA)—are central players in RNAi. Both siRNAs and miRNAs regulate gene expression by annealing to mRNA sequence elements that are fully or partially complementary [1, 2]. Since transfected synthetic siRNAs were shown to induce RNAi in mammalian cells [3], they have been widely used to decipher gene function through suppression of gene expression, and they have also attracted attention because of their potential for therapeutic applications [4, 5]. miRNAs are a phylogenetically conserved family of endogenous small RNAs that play important roles in a wide variety of biological functions, including cell differentiation, tumor genesis, apoptosis, and metabolism [1, 2, 6, 7].

miRNAs are initially generated as long primary transcripts (pri-miRNA) that are processed in the nucleus by the enzyme complexes Drosha and DiGeorge Critical Region 8 (DGCR8) to a 70–90 nt stem-loop structure called pre-miRNA. The pre-miRNA is then exported to the cytoplasm. There, the exported pre-miRNA or exogenous dsRNA is cleaved by the enzyme Dicer into a ~22-nucleotide (nt) duplex known as miRNA or siRNA, respectively. The duplex is then incorporated into the RNA-induced silencing complex (RISC). After removing one strand called the passenger strand, the remaining strand, called the guide strand, in the RISC guides the silencing complex to target mRNAs. Thus, downstream of their initial processing, siRNAs and miRNAs share the same cellular machinery [1, 2].

Understanding the mechanism of the RNAi pathway has led to the development of alternative approaches of RNAi. Several groups have developed artificial miRNAs in the form of short-hairpin structures, called shRNAs, instead of siRNAs [8, 9]. DNA vector-based shRNAs are widely used. By contrast, the use of synthetic shRNAs is very limited [10–12], although synthetic shRNAs can easily incorporate chemical modifications to improve their stability and biological activity, similar to synthetic siRNAs. Probably, this limited use is because the chemical synthesis of long RNA oligonucleotides is generally difficult, consequently leading to high cost and low yield. Unlike DNA oligonucleotides, synthesis of RNA
2. Materials and Methods

2.1. Synthetic RNA Oligonucleotides. The siRNA sequence targeting LMNA (GenBank accession number: NM_170707) was from position 600–620 relative to the start codon. The shRNAs in Figure 1(a) were kindly provided by Nippon Shinyaku Co., Ltd. (Kyoto, Japan). These RNA oligonucleotides were synthesized as previously described [14]. To anneal shRNAs, shRNAs suspended in water were incubated for 5 min at 95°C in a heat block and then left until the block reached 25°C according to the manufacturer's protocol. Control (siGENOME Non-Targeting siRNA no. 2, denoted as “ctrl”) and custom designed siRNAs for LMNA (Sigma-Aldrich, St. Louis, MO, USA), 40 μM R Ribonuclease Inhibitor (Takara Bio Inc., Shiga, Japan) and 1% Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan). FLAG-hAgo2 was immunoprecipitated with anti-FLAG M2 agarose beads (Sigma-Aldrich), and the beads were washed three times with lysis buffer and then directly suspended in the RNA extraction reagent ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) to elute immunoprecipitated samples containing RNAs. RNA eluted in Isogen was isolated according to the manufacturer’s protocol.

2.2. Plasmid Construction. Human Ago2 (hAgo2) cDNA was amplified by PCR using the primers 5′-GGATCCATG-TACCTCGGGAGCAGGC-3′ and 5′-GCGGCGCTCAAA-GCAAGTACATGGTG-3′ after reverse transcription from total RNA isolated from HeLa cells and cloned into the pCR-blunt vector (Invitrogen, Carlsbad, CA, USA). A KpnI and NotI fragment containing a FLAG-tag coding region in the pcDNA3FLAG vector (kindly gift from E. Nishida, Kyoto University, Japan) was ligated with the KpnI and NotI-digested expression vector pcDNA5FRT (Invitrogen), yielding the pcDNA5FLAG vector. A BamHI and NorI-digested pcDNA5FRT vector was ligated with BamHI and NorI-digested pcDNA5FLAG vector, yielding the pcDNA5FLAG-hAgo2 plasmid. An EcoRI site was introduced into the XbaI site of the luciferase reporter vector pGL4.13 (Promega, Madison, WI, USA) by ligation with the oligonucleotides 5′-CTAGACT-GTAATTG-3′ and 5′-CTAGACTGTAATTG-3′, yielding the pGL4.13EcoRI vector. The oligonucleotides 5′-CTAGAG-AAGGAGGAACTGGACCTCGAG-3′ and 5′-AATTCGAG-ACTCCTGCTCCTCGTCT-3′ were annealed to form a dsDNA fragment, which was ligated with XbaI and EcoRI-digested pGL4.13EcoRI to produce the pGL4-LMNA plasmid. The identity of all constructs was confirmed by DNA sequencing.

2.3. Cell Culture and Transfection. A HEK293 line stably expressing FLAG-hAgo2 was established by using the Flp-In Expression System (Invitrogen) with the pcDNA5FLAG-hAgo2 plasmid and Flp-In-293 Cell Line (Invitrogen) according to the manufacturer’s protocol. HeLa cells and HEK293-derivative cells were maintained in DMEM with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin for both cell types, plus 100 μg/mL Hygromycin B for HEK293-derivative cells). Synthetic small RNAs were transfected by using X-tremeGENE siRNA Transfection Reagent (Roche, Mannheim Germany). Reporter plasmids were transfected by using FuGENE 6 Transfection Reagent (Roche). HeLa cells were plated in 24-well plates (3 × 10^4 cells/well) and in 12-well plates (6 × 10^4 cells/well), and HEK293-derivative cells were plated in 10 cm dishes (2 × 10^5 cells/dish) 24 h before transfection.

2.4. RNA Extraction and qRT-PCR Analysis. Total RNA was isolated from cultured cells with an RNeasy mini kit (QIAGEN, Hilden, Germany). To isolate RNA from immunoprecipitated hAgo2 protein, cells were lysed in 20 mM HEPEs (pH 7.4), 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 1 mM DTT, 1% EMPIGEN BB detergent (Sigma-Aldrich, St. Louis, MO, USA), 40 U/mL R Ribonuclease Inhibitor (Takara Bio Inc., Shiga, Japan) and 1% Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan). FLAG-hAgo2 was immunoprecipitated with anti-FLAG M2 agarose beads (Sigma-Aldrich), and the beads were washed three times with lysis buffer and then directly suspended in the RNA extraction reagent ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) to elute immunoprecipitated samples containing RNAs. RNA eluted in Isogen was isolated according to the manufacturer’s protocol.

To analyze mRNA expression, a QuantiTect Reverse Transcription Kit (QIAGEN) and a Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) were used. To quantify the amount of antisense and sense strand in the RISC, a miScript PCR System (QIAGEN) was used. The primers for PCR analysis were 5′-GGAGTCTGAGTTCTCTCTTC-3′ for the LMNA antisense strand, 5′-GAAAGGAGGAACTGGACCTCCA-3′ for the LMNA sense strand, 5′-GAGGAGGCTGGACCTCCA-3′ for the LMNA sense strand with a deletion, and 5′-TAGCTTATCAGA-CTGATGTTG-3′ for miR-21; 5′-AGTCCATTCAAGACAT TGGGAG-3′ and 5′-GGTGAGATGAAGGTGAGCAG-3′ for IFIT1; and 5′-CAACCATGAGTACAAATGGTG-3′ and 5′-CTAGTATGTGTATCCTCCA-3′ for IFIT1.

2.5. Immunoblotting and Reporter Analysis. For the immunoblotting assay, HeLa cells plated on 12-well plates were transfected with 5 pmol of RNA oligonucleotide. After 48 h, the cells were harvested by scraping them from culture dishes into hot 1× SDS sample buffer, and the lysates were separated by SDS-PAGE and analyzed by immunoblotting. The immunoblots were visualized and quantified by using an LAS-3000 imaging system (Fujifilm, Tokyo, Japan) and normalized to the levels of β-actin. Anti-Lamin A/C rabbit IgG (no. 2032) was purchased from Cell Signaling (Danvers, MA, USA). Anti-Lamin A rabbit IgG (L1293) and β-actin monoclonal mouse IgG (clone A-15) were from Sigma-Aldrich.
The abbreviation for transfection.

shRNAs targeting LMNA was analyzed by immunoblotting. TF is the human pre-miR-155 sequence. (b) The RNAi activity of these shRNAs targeting LMNA mRNA sequence. The lower cases are derived from WT Expression Kit (Ambion) according to the supplier’s protocol. HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) were hybridized with 11μg of labeled cRNA, washed, stained, and scanned according to the protocol described in the Affymetrix GeneChip Expression Analysis Manual. Affymetrix data were extracted, normalized, and summarized with the robust multiaverage (RMA) method Expression Console.

3. Results

3.1. Design of Pre-miRNA-Based shRNAs. To assess the activity of synthetic shRNAs, we selected Lamin A/C (LMNA) as an RNAi target because this gene has been widely used as a positive control in siRNA experiments. Previous reports have developed miR-155-based vectors for RNAi [15, 16]; thus, we designed four shRNAs modeled on the pre-miRNA of human miR-155 (Figure 1(a)). All of the designed shRNAs had the same target site of the LMNA coding region but slightly different structures. The 17 nt loop structure and the 2 nt 3' overhang were common parameters and are present in human pre-miR-155. It is known that Dicer efficiently cleaves dsRNA with a 2 nt 3' overhang [11, 17] and that most pre-miRNAs have a 2 nt 3' overhang generated by Drosha cleavage [18]. Two shRNAs (shRNA no. 1 and shRNA no. 1b) had a 24 bp stem length with or without an internal bulge, the other two (shRNA no. 2 and shRNA no. 2b) had a 21 bp stem length, generated by simply shortening the corresponding 24 bp stem shRNA at the loop side. Unlike in a previous report [11], the antisense strands were positioned 5' to the loop. This position ensured that the antisense strand had a fixed 5' end, irrespective of the position of cleavage by Dicer. The internal bulges in the stem region were introduced by a 2 nt deletion in the 3' arm sequence mimic the structures of pre-miRNAs. These shRNAs were tested for their ability to silence the endogenous LMNA gene. HeLa cells were transfected with each shRNA, and the expression of LMNA protein was analyzed by immunoblotting. All four shRNAs knocked down expression of LMNA with similar efficiency (Figure 1(b)).

3.2. Potent RNAi Activity of Synthetic shRNA. Next, we compared the activity of siRNAs and shRNAs, by synthesizing siRNAs with same target sequence as the shRNAs. Immunoblotting analysis showed that shRNA no. 2 and shRNA no. 2b had RNAi activity comparable to that of the corresponding siRNA (Figure 2(a)). When shRNA no. 1 and shRNA no. 1b were used, essentially the same results were obtained (data not shown).

To analyze the activity more quantitatively, a more sensitive assay was conducted. We generated a luciferase construct that contained a perfectly complementary target site in its 3'-UTR and evaluated the reporter activity in cells cotransfected with various concentrations of synthetic small RNAs. All shRNAs, at all concentrations, were a 2- to 3-fold more potent RNAi trigger than the corresponding siRNA (Figure 2(b)). Importantly, even at the lowest concentration (0.4 nM), all shRNAs had silencing activity comparable to

For the reporter assay, HeLa cells plated in 24-well plates were transfected first with RNA oligonucleotide (0.25, 1.25, 2.5 pmol) 24 h before harvesting and then with reporter plasmid (100 ng/well reporter luciferase plasmid, pGL4-LMNA, and 25 ng/well renilla luciferase vector, pGL4.73; Promega) 30 min later. The luciferase activity was measured by using a dual-luciferase reporter assay system (Promega) with a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). As an internal control, renilla luciferase activity was used. The data reported represent the means and standard deviations of three independent experiments.

2.6. Microarray Analysis. HeLa cells plated in 12-well plates were transfected with 5 pmol of RNA oligonucleotide, and RNA was extracted 48 h after transfection. 100 ng of total RNA was amplified and labeled by using an Ambion RNA was amplified and labeled by using an Ambion Expression Console.
that observed at their 10-fold higher concentration (4 nM). These results are coincident with previous reports [11, 19] and indicate that synthetic shRNAs are more potent than their corresponding siRNAs. In this assay, we could not detect significant differences among the four shRNAs tested.

### 3.3. Efficiently Incorporation of Synthetic shRNAs into the RISC.

As previously discussed [11], we reasoned that the more effective RNAi achieved by shRNAs as compared with siRNAs might reflect a difference in the efficiency of their incorporation into the RISC. We therefore examined the amount of antisense strands in the RISC generated from transfected small RNAs. Two shRNAs (no. 1 and no. 1b) were transfected into HEK293 cells stably expressing FLAG-tagged hAgo2 protein, which is a core component of the RISC [20]. Processed RNAs loaded into the RISC were isolated by coimmunoprecipitation with FLAG-tagged hAgo2 protein. As expected, real-time quantitative RT-PCR analysis revealed that the antisense strands derived from shRNAs were more efficiently incorporated into the RISC as compared with those derived from the corresponding siRNA (Figure 2(c)). Presumably, this efficient incorporation into the RISC underlies the highly potent silencing activity of shRNAs.

Interestingly, introducing an internal bulge in the stem increased the amount of antisense strands (compare no. 1b with no. 1 in Figure 2(c)). This might indicate that the equilibrium between the sense and antisense strands shifted towards antisense loading. We tested this equilibrium shift by quantifying the amount of antisense strands in the RISC. Indeed, introducing an internal bulge changed the equilibrium toward antisense loading (Figure 2(d)).

### 3.4. Off-Target Effects and Interferon Responses Induced by Synthetic shRNAs in HeLa Cells.

It is known that siRNAs can cause off-target effects [21]. Using microarray gene expression profiling, we compared the off-target effects of our synthetic small RNAs. From gene expression profiling, we identified genes that showed a more than 2-fold decrease in expression as compared with the control siRNA transfected sample. A Venn diagram of these downregulated genes is shown in Figure 3. As compared with siRNA transfection...
of shRNAs caused a broader downregulation of nontargeted transcripts. This might be simply because of the efficient incorporation of synthetic shRNAs into the RISC and the consequent higher potent RNAi activity (Figure 2(c)). We noted that introducing a bulge decreased the number of off-target genes (compared no. 2b with no. 2). This phenomenon might reflect preferential incorporation of the antisense strand into the RISC, as occurred when shRNAs with a bulge were used (Figure 2(d)).

Next, we examined the expression of interferon-related genes among the microarray data and found that IFIT1 [22] and IRF9 [23] were induced more than 2-fold by transfection of shRNA no. 2. We further analyzed the expression of these two genes by qRT-PCR analysis (Figure 4). Transfection of the siRNA upregulated IRF9 and IFIT1 expression by approximately 1.5-fold; however, transfection of shRNAs without a bulge caused slightly higher upregulation of these genes. Again, shRNAs with a bulge improved interferon responses. IFIT1 is known to be the mRNA that is most strongly induced in response to dsRNAs [22]. Introducing a bulge into the stem could circumvent this immune activation.

4. Discussion

In this study, we demonstrated that synthetic pre-miRNA-based shRNAs have more potent RNAi activity than their corresponding siRNA and found that their antisense strands are more efficiently incorporated into the RISC. A previous study also showed that synthetic shRNAs with a 27 nt stem and a 4 nt artificial loop have a significantly higher gene-silencing activity than conventional 21 nt siRNAs [11]. The authors demonstrated that these small RNAs are subjected to Dicer processing both in vitro and in vivo and speculated that siRNA duplexes generated by Dicer are efficiently loaded into the RISC. In a study using DNA vector-based shRNAs, two types of shRNA construct modeled on pre-miRNA and pri-miRNA transcripts were examined [24]. The results indicated that RNAi triggers that enter the RNAi pathway by a more natural route yield more effective silencing [25]. Here we demonstrated the efficient incorporation of shRNAs into the RISC, and our results are highly consistent with those of previous studies. Furthermore, introducing a bulge enhanced incorporation into the RISC and shifted the equilibrium toward antisense loading (Figures 2(c) and 2(d)). However, we did not observe a significant difference in RNAi activity among the synthetic shRNAs in our assay (Figure 2(b)), probably because they were all close to saturation.

There are contradictory reports about the effects of stem length on RNAi activity. Some reports have shown that shRNAs with a 19 nt stem and a 9 or 10 nt loop have higher RNAi activity than shRNAs with longer stem [12, 26, 27]. This type of shRNA is widely used as a vector-based shRNA [28]. Interestingly, shRNAs with a 19 nt stem are not Dicer substrates; however, these shRNAs are thought to be incorporated into the RISC after bypassing Dicer processing [11]. Although we reasoned that the development of shRNAs based on naturally found structures is a promising approach, we should also examine and evaluate this type of shRNA. Unlike previously used shRNAs [11], our designed shRNAs have a shorter stem length (21 or 24 nt) and the loop derived from a pre-miRNA and are therefore expected to be less toxic. However, we observed that interferon responses were induced more strongly by shRNAs than by their corresponding siRNA. Interestingly, introducing a bulge also
reduced interferon responses. These observations, albeit of a few examples, indicate that introducing a bulge has potential for improving the performance of shRNAs. Further examination of the position and structure of bulges is required. Chemical modifications of siRNAs have been extensively studied to increase stability, promote efficacy, minimize off-target effects, and reduce innate immune responses [5, 21]. Such modifications can be easily incorporated in synthetic shRNAs to improve siRNA stability and biological activity, similar to synthetic siRNAs. These are issues to be addressed in the future.

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References


Research Article

Conformational-Dependent and Independent RNA Binding to the Fragile X Mental Retardation Protein

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1. Introduction

Fragile X syndrome is the most common inherited cause of mental impairment accounting for ca. 40% of X-linked mental retardation cases. It is also the most common known cause of autism (reviewed in [1–6]). Other characteristics of the fragile X syndrome include hyperactivity [7], increased susceptibility to seizures [8], increased testicular volume [9], macrocephaly, and large ears [10]. In addition, it has been found that carriers of the fragile X premutation, once thought to be free of the effects of the disease, also suffer from subtle behavioral and physical abnormalities [11–14]. This wide and varied constellation of phenotypic features results from the loss of function of a single gene, FMR1 (summarized in: http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=atlas&part-fragilex).

The FMR1 gene encodes the RNA-binding protein FMRP [15], a negative [16–18] and positive [19, 20] translational regulator, and it has been of considerable interest to delineate the cellular RNAs that bind to FMRP [21–25] and the mechanism(s) by which FMRP binds and controls these mRNAs [26–35].

In 2003, Zalfa et al. described a bridging mechanism in which the fragile X mental retardation protein (FMRP) via interaction with the 5′ end of the small noncoding RNA, BC1, and bound and repressed FMRP target mRNAs [36]. This model has been subject to great deal of scrutiny owing to findings that appear to be out of step with other studies. These include differences in the prime localization of FMRP with small repressed mRNP rather than brain polyribosomes [18, 37–39], differences in the interpretation of the interaction of FMRP with BC1 RNA as specific and significant [40] rather than nonspecific and insignificant [30, 41, 42] and detailed mechanistic differences in the nature of BC1 RNA-mediated localization and translational repression [41, 43–45]. In response to some of these criticisms, Zalfa
and Bagni reposed that their model, rather than being a general model of FMRP-mRNA interactions, was only one of several possible models [46].

More recent investigations of some of the concomitants of the Zalfa model [47] found that the interaction of recombinant FMRP with BC1 RNA was weak compared to that of a G-quartet-containing RNA. Furthermore, its strength varied significantly depending upon the buffer conditions used. The data suggested that FMRP may interact with a particular conformer of BC1 RNA. Here, we elaborate the conditions and requirements for a weak FMRP BC1 RNA interaction in vitro.

2. Materials and Methods

2.1. Buffers. Z-buffer is 10 mM Tris-HCl pH 7.5, 2 mM MgCl2, 400 mM NaCl and 0.2% SDS [36]. The RNA-binding buffer that was used for affinity capture is 50 mM Tris-HCl, pH 7.4, 2 mM MgCl2, and 150 mM KCl, 1 mM EDTA, and 1 mM DTT [26]. Structure buffer is 10 mM Tris-HCl pH 7, 100 mM KCl, and 10 mM MgCl2.

2.2. Preparation of BC1 RNA Transcripts. Dra I linearized pBCX607 containing the entire BC1 sequence [48], AatII linearized pBCX607 containing the first 65 b of BC1, Sac I linearized pMK-1 containing the last 60 b of BC1 RNA, a PCR fragment encoding a T7 RNA polymerase promoter and the first 75b of BC1 RNA. Hind III linearized pTAR encoding an 85b transcript that folds into a 57 b TAR element and a 28 b leader sequence and linearized pTri-XEF1 encoding eEF-1A mRNA (Ambion) were used to produce biotinylated RNAs via in vitro transcription (Ambion). Plasmids pBCX607 and pMK-1 were provided by Dr. Henri Tiedge (SUNY Brooklyn). Alcohol-precipitated RNAs were dissolved in 50 μL DEPC-treated H2O and quantified spectrophotometrically. RNA integrity was examined by agarose gel electrophoresis. For examining the effect of annealing on RNA-protein interactions, individual RNAs were heated in 1x transcription buffer (Ambion) at 65°C for 10 min and then allowed to cool slowly to room temperature for one hour prior to their use. Note. We obtained identical results with different protein variants [57, 58].

2.3. RNA-Binding Assays. 35S-FMRP, 35S-FMRP280, 35S-FMRP204, 35S-FXR1P, 35S-FXR2P, 35S-eIF4A, and 35S-luciferase were and were produced from plasmids pET21A-FMRP, pND-L-mHisFMRP, pET9-FMRP280, pET9-FMRP204, pHA-FXR1P, and pET21b-FXR2P in an RRL-coupled transcription-translation system (Promega). Plasmid pHA-FXR1P was provided by Dr. Gideon Dreyfuss (University of Pennsylvania); plasmids pET9-FMRP280 and pET9-FMRP204 were provided by Dr. Darryl Spinner (IBR); plasmid pET21b-FXR2P was a gift from Dr. Jennifer Darnell (Rockefeller University) and plasmid pET-His6-eIF4A was a gift from Dr. Henri Tiedge (SUNY Brooklyn). Briefly, 35S-labeled proteins were produced by combining twenty five microliters of TNT rabbit reticulocyte lysate (RLR) with 2 μL of TNT T7 RNA polymerase, 1 μL of 1 mM amino acid mix minus-methionine, 35 μCi of 35S-methionine, 1 μL of RNAsin and 1 μg of plasmid DNA in a total volume of 50 μL. One microliter of a 50X Complete protease inhibitor cocktail was added to prevent proteolysis. Samples were incubated at 30°C for 90 min and then assayed for protein production by autoradiography.

Affinity capture assays were performed as described previously [17]; the bound and unbound products were resolved by SDS-PAGE and subject to autoradiography. Autoradiograms were quantified using UN-SCAN-IT Gel 6.1 (Silk Scientific, Inc.). The percent binding was calculated as

\[
\text{%Binding} = 100 \times \frac{\text{Intensity}_{\text{bound}}}{\text{Intensity}_{\text{bound}} + \text{Intensity}_{\text{unbound}}}. \quad (1)
\]

The percent binding of the “no RNA” control in each experimental set was subtracted from that of the samples; the difference, representing authentic binding, was plotted.

2.4. RNA Structure Studies. Annealed and unannealed BC1 RNA, BC1 fragment RNAs and control RNAs (1-2 μg), were treated at room temperature for 15 min with various amounts of ribonuclease V1 (cobra venom) or ribonuclease A in structure buffer as indicated. Reaction products were resolved on 1-2% TAE agarose gels containing 0.1 μg/mL ethidium bromide along with appropriate size markers. Gels were imaging using a Scion CFW-1308 M mega pixel camera and captured in inverted mode using FOTO/Analyst PC Image software version 9.04 (FOTODYNE). The resulting image files were digitized and analyzed using UN-SCAN-IT Gel 6.1.

2.5. RNA Secondary Structure Modeling. RNA lowest energy secondary structures were determined using the Zuker algorithm, M-fold (http://mfold.rna.albany.edu).

3. Results and Discussion

3.1. Affinity Capture of FMRP with Biotinylated RNA. Several methods have been used to assess the direct physical interaction of FMRP with RNA in vitro. These include pull-down assays with homoribopolymers [15, 21, 49, 50], affinity capture using biotinylated RNA [21, 41, 51–53], affinity capture using immobilized protein [16], UV crosslinking [21, 49], filter-binding assays [22, 30, 41], electrophoretic mobility shift assays (EMSA) [26, 36, 40, 42, 47, 54], and agarose electrophoretic mobility shift assays (AGESA) [17, 41]. Each of these methods has its unique experimental advantages [55]. Acknowledging that binding between a nucleic acid and RNA-binding protein (RBP) can be affected by differences in posttranslational modification [56] and/or differences between different protein variants [57, 58], our working hypothesis is that given a particular RBP, a particular RNA and a defined buffer each of these methods should converge to produce a common answer. While extensively studied, FMRP’s interactions with RNA have not always been examined with this hypothesis in mind Table 1, and it has been suggested that differences in experimental conditions.
and protein preparations form the basis for the divergent results obtained for the FMRP BCI RNA interaction [59, 60]. We chose to examine heretofore unstudied aspects of FMRP’s interaction with BCI RNA using affinity capture, one of the older techniques used in FMRP RNA binding studies. We next sought a basic buffer to use. In several previous publications, we have used a buffer described by Schaeffer et al. [26], which used physiological saline [17, 41, 47], Table 1. However, this buffer contains tRNA to reduce nonspecific binding, and as two publications demonstrate that FMRP can interact with tRNA [42, 47], we first determined whether specific binding between FMRP and RNA could be observed without the addition of tRNA. As shown in Figure 1 (upper panel), in the presence of physiological salt and in the absence of RNA, 35S-FMRP translation in rabbit reticulocyte lysate (RRL) nonspecifically bound to the avidin affinity column; however, addition of increasing amounts of NaCl decreased this nonspecific interaction so that at 125 mM NaCl the amount of bound FMRP was between 5%–10% of the total. As expected, tRNA also blocked the association of full-length RRL nonspecifically bound to the avidin affinity column; Figure 1 (lower panel). Thus, tRNA, 0.01% BSA, 8 U of RNasin.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Assay</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ashley et al. (1993) [51]</td>
<td>Pull-down</td>
<td>16 mM HEPES-KOH pH 7.9, 120 mM KCl, 0.04% Nonidet P-40, 1 mg/mL BSA, 0.16 mM dithioerythritol, 0.4 mM phenylmethylsulfonyl fluoride</td>
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<tr>
<td>Brown et al. (1998) [49]</td>
<td>Pull-down</td>
<td>10 mM Tris-HCl pH 7.5, 2.5 mM MgCl2, 100 mM NaCl, 2.5% Triton X100, 1 mg/mL heparin</td>
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<tr>
<td>Price et al. (1996) [52]</td>
<td>Pull-down</td>
<td>20 mM Heps, pH 7.9, 2 mM MgCl2, 10 mM ZnCl2, 70 mM NH4Cl, 0.02% Nonidet P-40, 5 mg/mL yeast tRNA</td>
</tr>
<tr>
<td>Sung et al. (2000) [21]</td>
<td>Pull-down</td>
<td>20 mM Heps, pH 7.9, 2 mM MgCl2, 10 mM ZnCl2, 70 mM NH4Cl, 0.02% Nonidet P-40, 5 mg/mL yeast tRNA</td>
</tr>
<tr>
<td>Denman and Sung (2002) [57]</td>
<td>Pull-down</td>
<td>20 mM Heps, pH 7.9, 2 mM MgCl2, 10 mM ZnCl2, 70 mM NH4Cl, 0.02% Nonidet P-40, 5 mg/mL yeast tRNA</td>
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<tr>
<td>Schaeffer et al. (2001) [26]</td>
<td>EMSA</td>
<td>50 mM Tris-HCl pH 7.4, 1 mM MgCl2, 1 mM EDTA, 150 mM KCl, 1 mM DTT, 0.25 mg/mL of E.coli tRNA, 0.01% BSA, 8 U of RNasin</td>
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<tr>
<td>Sung et al. (2003) [17]</td>
<td>Pull-down</td>
<td>50 mM Tris-HCl, pH 7.0, 2 mM MgCl2, 150 mM NaCl, 1 mM DTT, 0.25 mg/mL E.coli tRNA, 0.25 mg/mL BSA</td>
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<tr>
<td>Bechera et al. (2006) [61]</td>
<td>EMSA</td>
<td>50 mM Tris-HCl pH 7.4, 1 mM MgCl2, 1 mM EDTA, 150 mM KCl, 1 mM DTT, 0.25 mg/mL of E.coli tRNA, 0.01% BSA, 8 U of RNasin</td>
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<tr>
<td>Didiot et al. (2008) [54]</td>
<td>EMSA</td>
<td>50 mM Tris-HCl pH 7.4, 1 mM MgCl2, 1 mM EDTA, 150 mM KCl, 1 mM DTT, 0.25 mg/mL of E. coli tRNA, 0.01% BSA, 8 U of RNasin</td>
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<td>Zalfa et al. (2003) [36]</td>
<td>EMSA</td>
<td>10 mM HEPES pH 7.9, 3 mM MgCl2, 10 mM DTT, 100 mM KCl, 750 mM NaCl, 5% glycerol, 7 mM β-Mercaptoethanol, 1 mg/mL Albumin, 1.3 mg/mL Heparin</td>
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<td>Zalfa et al. (2005) [40]</td>
<td>EMSA</td>
<td>20 mM HEPES-KOH, pH 7.6, 5 mM MgCl2, 300 mM KCl, 2 mM DTT, 5% glycerol, and 500 ng of total yeast tRNA or 20 µg of heparin</td>
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<td>Darnell et al. (2001) [22]</td>
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<td>Darnell et al. (2005) [30]</td>
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<td>50 mM Tris-OAc at pH 7.7, 50 mM KOAc, 10 mM Mg(OAc)2, 30 μg/mL tRNA</td>
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<td>Gabus et al. (2004) [42]</td>
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<td>20 mM Tris-HCl pH 7.5, 30 mM NaCl, 0.2 mM MgCl2, 5 mM DTT, 10 μM ZnCl2</td>
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<tr>
<td>Laggerbauer et al. (2001) [16]</td>
<td>Pull-down</td>
<td>PBS, 0.02% IGEPAL, 1% BSA</td>
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<tr>
<td>Siomi et al. (1993) [15]</td>
<td>Pulldown</td>
<td>10 mM Tris-HCl pH 7.4, 2.5 mM MgCl2, 0.5% Triton X-100, 100–1000 mM NaCl</td>
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<td>Steitler et al. (2005) [56]</td>
<td>Pulldown</td>
<td>2 M KOAc, 100 mM Tris-OAc pH 7.7 and 50 mM MgOAc2, 1 μL of yeast tRNA, 1 μL of RNasin</td>
</tr>
<tr>
<td>Menon and Mihaiilescu (2007) [62]</td>
<td>EMSA</td>
<td>50 mM Tris-HCl pH 7.5, 150 mM NaCl and protease inhibitors</td>
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<tr>
<td>Fahling et al. (2009) [20]</td>
<td>EMSA</td>
<td>10 mM Heps pH 7.2, 3 mM MgCl2, 5% glycerol, 1 mM DTT, 150 mM KCl, 2 U/μL RNaseOUT, 0.5 μg/μL rabbit tRNA</td>
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<tr>
<td>Zou et al. (2008) [63]</td>
<td>AGESA</td>
<td>20 mM Tris-HCl pH 7.2, 150 mM NaCl</td>
</tr>
<tr>
<td>Iacoangeli et al. (2008) [47]</td>
<td>EMSA</td>
<td>50 mM Tris-HCl pH 7.6, 150 mM KCl, 1 mM MgCl2, 1 mM EDTA, 1 mM DTT, 0.2 U/μL RNase inhibitor, 100 ng/μL total yeast tRNA, and 100 ng/μL BSA</td>
</tr>
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</table>

Sets of binding conditions are grouped by the different laboratories that used them. Each set of conditions is differentiated from the next by a dotted line. In some instances the same group used multiple sets of binding conditions in multiple publications.
Figure 1: NaCl blocks nonspecific binding of FMRP with avidin. (a) 35S-FMRP was bound to SoftLink resin in 1x Schaeffer binding buffer supplemented with 0 mM, 125 mM, 250 mM and 375 mM NaCl, lanes 2–5, respectively. Bound 35S-FMRP was recovered, resolved by SDS-PAGE and subject to autoradiography. Lane 1 shows the amount of 35S-FMRP input into the assay. (b) 35S-FMRP was bound to SoftLink resin in 1x Schaeffer binding buffer supplemented with 0.05 mg/mL, 0.1 mg/mL, 0.15 mg/mL, and 0.25 mg/mL tRNA, lanes 2–5, respectively. Bound 35S-FMRP was recovered, resolved by SDS-PAGE and subject to autoradiography. Lane 1 shows the amount of 35S-FMRP input into the assay. The asterisk marks a 35S-truncation product produced by transcription/translation.

Schaeffer buffer supplemented with 125 mM NaCl blocks the non-specific interaction of FMRP with the affinity matrix as effectively as Schaeffer buffer supplemented with tRNA.

3.2. Prior Annealing of BC1 RNA Enhances Its Interaction with FMRP. Having established this basic set of conditions, we applied them to study the FMRP BC1 RNA interaction. The weak binding between FMRP and BC1 RNA that occurs in protein excess [36] suggested that the protein might be surveying the population of BC1 RNA conformers and interacting with a particular one. As BC1 RNA is known to form higher order structure [45], we performed a simple experiment to perturb the conformer population that would test this hypothesis. BC1 RNA was transcribed in vitro and purified by salt and alcohol precipitation; then, it was either heated briefly or left untreated. Each RNA (annealed and unannealed, resp.) was then was titrated with a constant amount of 35S-FMRP, and the binding was assessed by affinity capture. Under the conditions used, unannealed BC1 RNA bound extremely weakly at all concentrations examined, in concert with previous obtained results [41, 64]. On the other hand, annealed BC1 RNA exhibited stronger binding to FMRP over the range of concentrations examined, Figures 2(a) and 2(b).

To index the above results to a known standard, the interaction between eEF-1A mRNA and FMRP was also measured in parallel. Previous work had shown that this RNA binds strongly to FMRP, without prior annealing [17, 41]. As expected, the FMRP eEF-1A mRNA interaction was much stronger than the FMRP BC1 RNA interaction, Figure 2(c). In fact, with the same amount of 35S-FMRP, unannealed eEF-1A mRNA evinced saturable binding at 90 nM RNA, while it took 100-fold more annealed BC1 RNA to achieve comparable binding; see Figure 2(d).

To determine whether annealing affected the interaction of FMRP with eEF-1A mRNA, binding to annealed and unannealed forms of the message was assessed using a subsaturating concentration of eEF-1A RNA. As shown in Figure 2(e), binding of eEF-1A mRNA to 35S-FMRP was not markedly affected by annealing.

3.3. Annealing Alters the Structure of BC1 RNA. The above data implied that the conformer populations of annealed and unannealed BC1 RNA differ. To determine whether this was so, the annealed and unannealed BC1 RNAs used in Figure 2 were treated with RNases whose cleavage depends on known RNA structural features and the products were resolved on nondenaturing agarose gels. In the absence of treatment, both RNAs displayed a major band as well as a less prominent, slower migrating band, Figure 3(b). These data indicate that annealed BC1 RNA and unannealed BC1 RNA contained multiple conformers. Interestingly, in the presence of RNase A, which preferentially cleaves at single-stranded C and U residues, both annealed and unannealed BC1 RNA behaved nearly identically and were completely degraded by all but the lowest amount of the enzyme. However, when the RNAs were treated with a range of concentrations of RNase V1 (0.01–1 units), which cleaves base-paired nucleotides, the unannealed form was refractory to cleavage, while the annealed form was sensitive toward cleavage at the highest amount, Figure 3(c) (upper panel). Increasing the amount of RNase V1 recapitulated the sensitivity of annealed BC1 RNA, Figure 3(c) (lower panel); however, it also demonstrated that a fraction of unannealed BC1 RNA also contained RNase V1-sensitive stable duplex RNA. To determine whether the unique BC1 RNA conformer(s) produced by annealing could be stabilized by MgCl2 the annealing reaction was also carried out in the presence of 2 mM MgCl2. The results indicated that the magnesium did not alter the distribution of BC1
RNA conformer(s) or affect their resistance to RNase V1 (not shown).

These data unequivocally demonstrate that the conformer populations of annealed and unannealed BC1 RNA differ, and this difference is due to an increase in the amount of stable RNA duplexes in the annealed RNA. Moreover, the data support the hypothesis that the binding between FMRP and annealed BC1 RNA results from a unique conformer that is absent from the unannealed BC1 RNA conformer population.

3.4. Functional Dissection of the FMRP BC1 RNA Interaction.

BC1 RNA contains several distinct higher order structural elements. Its 5’ end contains two cis-acting spatial targeting elements, DTE1 and DTE2 [45]. The former is required for somatic export of BC1 RNA into dendrites, while the latter specifies long-range distal delivery and is mediated by a prominent GA-type kink turn (KT) motif in the apical region of the 5’ BC1 domain [45]. The 3’ 60 bases of BC1 RNA contains an abbreviated A-rich region and a 3’-terminal stem-loop structure [65] that has been shown to bind synergistically to the eucaryotic initiation factor 4A (eIF4A) and the polyadenylation binding protein (PABP) [41]. To further probe this interaction, BC1 RNA was functionally dissected, and these two elements were examined individually. As shown in Figure 4(a), RNase VI treatment of the 5’ 75 bases of BC1 RNA recapitulated the results of full-length BC1 RNA indicating that it forms a stable secondary structure in the absence of the 3’ end. This agrees with modeling studies using the Zuker M-fold.
algorithm, which show that the first 75 bases of BC1 RNA fold identically to that portion of the full-length molecule, Figure 4(b). On the other hand, annealing had no effect on the sensitivity of the 3′ 60 bases of BC1 RNA towards RNase VI, Figure 5. Thus, these data demonstrate that the difference in the conformer populations of annealed and unannealed BC1 RNA most likely arise from alterations in the 5′ end of the molecule.

We next examined whether FMRP preferentially bound either of the dissected RNAs. Therefore, 35S-FMRP was incubated with 2 μM annealed versions of each RNA; binding was subsequently assessed by affinity capture and compared to that of annealed full-length BC1 RNA. We found that the 5′ 75 bases of BC1 RNA bound slightly less than full-length BC1 RNA, Figure 6(a); however, the difference did not rise to the level of statistical significance (P = .15, ANOVA). Truncation of this RNA by a 15 base deletion at its 3′ end further decreased binding (P = .08, ANOVA). Significantly, this 60 base RNA is not expected to recapitulate the folding of the first 60 bases of full-length BC1 RNA, Figure 6(b). On the other hand, the 3′ 60 bases of BC1 RNA evinced no evidence of binding, Figure 6(a). These data suggest that the major determinant that FMRP recognizes in annealed BC1 RNA is the hairpin structure of the 5′ end. To test this further, we also conducted binding studies using an 85 base RNA harboring the HIV1 TAR hairpin, Figure 6(c). The results show that this RNA binds with the same affinity as annealed full-length BC1 RNA.

3.5. Specificity and Requirements of the FMRP BC1 RNA Interaction. Different preparations or sources of FMRP has been posited as a potential explanation for the divergent BC1 RNA-binding data obtained by different laboratories [59, 60] and a likely cause of such differences is in the posttranslational modifications that occur in each. To begin to address this question, we examined whether differences in posttranslational arginine methylation could alter the binding affinity of annealed BC1 RNA and FMRP. Specifically, we compared the
Figure 4: Functional dissection of structural elements in the 5′ end of BC1 RNA. (a) Ten-fold serial treatment of annealed and unannealed 5′BC1-75 RNA with RNase VI starting at 0.1 units/μL (upper panel) or 0.5 units/μL (lower panel). Arrow marks the major conformer. Lane M shows 100 bp molecular weight markers. (b) Secondary structure model of 5′BC1-75 RNA from M-fold.

Figure 5: Functional dissection of structural elements in the 3′ end of BC1 RNA. (a) Ten-fold serial treatment of annealed and unannealed 3′BC1-60 RNA with RNase VI starting at 0.1 units/μL (upper panel) or 0.5 units/μL (lower panel). Arrow marks the major conformer. Lane M shows 100 bp molecular weight markers. (b) Secondary structure model of 3′BC1-60 RNA from M-fold.
ability of full-length FMRP and the ability of an alternatively spliced variant FMRP<sub>Ex15c</sub> to bind 2 μM annealed BC1 RNA. Previous studies showed that full-length FMRP is readily methylated in its RG-rich region, while FMRP<sub>Ex15c</sub>, which lacks 25 amino acids upstream of the RG-rich region, is refractory to arginine methylation [66]. Figure 7(a) shows that binding of annealed BC1 RNA to FMRP<sub>Ex15c</sub> was less than it was to full-length FMRP, but the difference did not rise to statistical significance.

We next examined whether the binding between annealed BC1 RNA and FMRP was unique. As an initial indicator, 2 μM annealed BC1 RNA was incubated with equimolar amounts of fragile X family members, 35S-FMRP, 35S-FXR1P and 35S-FXR2P (full-length forms); subsequently, binding was assessed as previously described. As shown in Figure 7(a), both FXR1P and FXR2P bound as well as FMRP. To confirm and extend these results binding studies between annealed BC1 RNA and luciferase or eIF4A were performed. Luciferase does not contain any RNA-binding motifs and does not bind to poly (rA), poly (rG), or eEF-1A mRNA, Figure 7(b). Correspondingly, we found that luciferase did not interact with annealed BC1 RNA. On the other hand, the RNA helicase, eIF4A, bound weakly annealed BC1 RNA under these conditions, Figure 7(a). These data indicate that in addition to FMRP annealed BC1 RNA can interact interchangeably with at least three other RNA-binding proteins.

It has been previously proposed that BC1 RNA interacts with the N-terminal domain (NTD) of FMRP [40]. Nevertheless, Wang et al. did not observe an interaction between unannealed BC1 RNA and a 280 amino acid NTD construct, FMRP<sub>1−280</sub> [41]. To complete this analysis, we also assessed the interaction of annealed BC1 RNA with FMRP<sub>1−280</sub>. As shown in Figure 7(a), annealed BC1 RNA did not bind to 35S-FMRP<sub>1−280</sub>. To determine whether 35S-FMRP<sub>1−280</sub> lacked the ability to interact with RNA, we asked whether it could bind to homoribopolymers. As shown in Figure 7(c), 35S-FMRP<sub>1−280</sub> was unable to bind to poly(rA), poly(rG), or poly(rI:rC). These data suggest that 35S-FMRP<sub>1−280</sub> does not significantly interact with ribonucleic acids under the conditions used. Interestingly, however, while a shorter NTD, that is, 35S-FMRP<sub>1−204</sub>, was also unable to bind to annealed
Figure 7: Annealed BC1 RNA binds pleiotropically to the FXRPs. (a) Equimolar amounts of $^{35}$S-FMRP, $^{35}$S-FMRP$_{15c}$, $^{35}$S-FXR1P, $^{35}$S-FXR2P, $^{35}$S-eIF4A, $^{35}$S-FMRP$_{280}$, $^{35}$S-FMRP$_{204}$, and $^{35}$S-Luciferase were incubated with 2 $\mu$M annealed BC1 RNA. The percent binding corrected for background of 6 reactions per protein is plotted. The relative levels of FMRP and FMRP$_{15c}$, FMRP and FXR1P and FMRP and FXR2P were not significantly different ($P = .17$, $P = .67$ and $P = .27$, resp., ANOVA). However, the relative levels of FMRP and eIF4A and FMRP and NTD$_{204}$ were significantly different ($P = .038$ and $P = .007$, resp., ANOVA). Since no binding above background was observed for NTD$_{280}$ and Luciferase relative differences were not assessed. (b) Pull-down assays between $^{35}$S-luciferase and poly (rG), poly (rA), eEF1A RNA (90 nM) and annealed BC1 RNA (5 $\mu$M) showing the unbound (U) and bound (B) fractions. Binding in the absence of RNA is shown for the negative control. (c) Pull-down assays between $^{35}$S-NTD$_{280}$ or $^{35}$S-NTD$_{204}$ and poly (rG), poly (rA), poly (rI : rC) and annealed BC1 RNA (5 $\mu$M) showing the unbound (U) and bound (B) fractions. Binding in the absence of RNA is shown for the negative control.

BC1 RNA, Figure 7(a), it was able to bind specifically to poly(rG) and to a lesser extent, poly(rI : rC), Figure 7(c). Thus, annealed BC1 RNA does not interact significantly with the 204 N-terminal residues of FMRP.

4. Conclusions

disparate data obtained using electrophoretic mobility shift assays have been published concerning the binding of FMRP to BC1 RNA [36, 40, 47]. To investigate this discrepancy, we turned to an affinity capture assay to measure RNA binding to FMRP under the assumption that the results from different assays should converge to produce a common result. This particular assay was first described by Boehlens et al. and applied to the interactions of FMRP and nucleic acids by Ashley et al. [51, 67]. Here, we demonstrate that in 150 mM KCl and 125 mM NaCl, the assay has a dynamic range of RNA binding of at least two logs. Thus, it is able to distinguish high affinity binding from low affinity binding.
We specifically developed these assay conditions, which do not utilize tRNA as a general nonspecific binding competitor, because it had previously been demonstrated that both tRNA and BC1 RNA bind to FMRP in a low salt buffer with nearly identical dissociation constants ($K_{ds}$) [42], and because tRNA was shown to directly displace BC1 RNA from FMRP [47]. Thus, tRNA, while admittedly effective, may not be the best blocking reagent for examining FMRP’s interaction with BC1 RNA. On the other hand, FMRP’s association with polyribosomes [37, 38], translationally repressed ribosomes within neuronal granules [68], and its function in translational regulation [17, 19, 27] indicates that it operates in an environment with a relatively high local concentration of tRNA, which would ensure a direct competition with BC1 RNA. Indeed, previously published immunoprecipitation experiments, which are carried out in the presence of a large excess of tRNA (both added and from the endogenous tRNA present during cell lysis) and which fail to find BC1 RNA among the precipitated transcripts are entirely consistent with a direct competition between BC1 RNA and tRNA [47]. Here, we demonstrate that BC1 RNA produced simply by in vitro transcription does not interact with FMRP even in the absence of tRNA, confirming our previous results using different assays and different binding conditions [41]. Nevertheless, by artificially annealing the BC1 RNA transcript, we found that binding occurred although it was at least a hundred-fold weaker than that between FMRP and eEF-1A mRNA. Previous protocols examining the interaction between BC1 RNA and FMRP have not indicated that the RNA was pretreated or annealed before binding to FMRP was initiated [36, 40, 47]; therefore, it must be assumed that it was not, and hence, this treatment is unique to this work.

To try to understand the result, we first determined whether enhanced binding due to annealing was a general feature of FMRP’s interaction with RNA. A search of the current literature produced mixed results. Darnell et al., working with kissing complex RNA a small double hairpin with additional loop-loop tertiary interactions that binds to FMRP’s KH$_2$ domain, preannealed the RNA before binding was initiated [30]. Likewise, Ramos et al. and Zanotti et al. preannealed the synthetic G-rich RNA, sc1, to form G-quartets before binding to RGG box peptides [69, 70]. Thus, tRNA, while admittedly effective, may not be the best blocking reagent for examining FMRP’s interaction with BC1 RNA. On the other hand, FMRP’s association with polyribosomes [37, 38], translationally repressed ribosomes within neuronal granules [68], and its function in translational regulation [17, 19, 27] indicates that it operates in an environment with a relatively high local concentration of tRNA, which would ensure a direct competition with BC1 RNA. Indeed, previously published immunoprecipitation experiments, which are carried out in the presence of a large excess of tRNA (both added and from the endogenous tRNA present during cell lysis) and which fail to find BC1 RNA among the precipitated transcripts are entirely consistent with a direct competition between BC1 RNA and tRNA [47]. Here, we demonstrate that BC1 RNA produced simply by in vitro transcription does not interact with FMRP even in the absence of tRNA, confirming our previous results using different assays and different binding conditions [41]. Nevertheless, by artificially annealing the BC1 RNA transcript, we found that binding occurred although it was at least a hundred-fold weaker than that between FMRP and eEF-1A mRNA. Previous protocols examining the interaction between BC1 RNA and FMRP have not indicated that the RNA was pretreated or annealed before binding to FMRP was initiated [36, 40, 47]; therefore, it must be assumed that it was not, and hence, this treatment is unique to this work.

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We next determined whether annealed BC1 RNA was structurally altered from unannealed BC1 RNA. Indeed, we showed that the annealed form exhibited an increased susceptibility to cleavage by RNase VI. These data support the hypothesis that a particular BC1 RNA conformer; that is, one containing a unique double-stranded RNA structure was the true FMRP interactor. Functional dissection of BC1 RNA into either a 5’ ID element or a 3’ element demonstrated that the major determinant that FMRP binds to is located in the 5’ 75 bases of the molecule. As FMRP also binds to tRNA and TAR RNA, these data imply that FMRP can weakly recognize hairpin-containing RNAs.

Another difference between the published FMRP BC1 RNA interaction studies was that one group used full-length recombinant FMRP produced from baculovirus [36, 40], while the other group used full-length recombinant FMRP produced in E. coli [47]. A potential concern here is that each of these preparations may be differentially posttranslationally modified. Indeed, it is well known that FMRP is subject to posttranslational methylation of arginine residues in its RG-rich region [18, 31]. However, while protein arginine methyltransferases are present in Spodoptera frugiperda [72], the host cells used in baculovirus FMRP production [40], they are absent from E. coli [73, 74]. Thus, it is possible that the presence or absence of methyl-arginine residues might directly or indirectly affect an interaction between FMRP and BC1 RNA. In the present work, we opted to produce full-length FMRP in rabbit reticulocyte lysates (RRL), which generates partially methylated FMRP [66]. Again, we assumed that posttranslational modifications arising from source differences could be a modulating but not the determining factor in a putative FMRP BC1 RNA interaction. Precedence for this assumption may be seen in the work of Stetler et al. who showed that methylating FMRP decreased, but did not abrogate, the direct binding of two G-quartet RNAs to FMRP’s RG-rich region [56]. Consistent with the work using E. coli-purified FMRP [47] and with the work of Wang et al. who used unmethylated FMRP produced in cell-free wheat germ lysates to examine BC1 RNA binding [41], we observed minimal interactions between RRL-produced FMRP and unannealed BC1 RNA. Thus, methylation FMRP’s arginine residues does not enhance its affinity for unannealed BC1 RNA. Furthermore, although binding between annealed BC1 RNA and the methylation refractory form of FMRP (FMRP$_{15}$) was reduced compared to methylated FMRP, it was not a statistically significant reduction. Thus, methylarginine residues in FMRP’s RG-rich region are not absolutely required for this interaction either.

We also probed the exclusivity of FMRP’s interaction with annealed BC1 RNA. Here, we found that interactions of similar magnitude occur with fragile X paralogs FXR1P and FXR2P and to a lesser extent, eIF4A. These results demonstrate that BC1 RNA binds indiscriminately to RNA-binding proteins. Interestingly, contrasting results were recently obtained for kissing complex (kc) RNA [34], where it was shown that this RNA exclusively interacts with the
fragile X paralogs but not with other related KH domain-containing RNA-binding proteins.

As all three paralogs have highly homologous N-terminal sequences but are more divergent in their C-terminal ends [75], we inquired whether annealed BC1 RNA interacted with specific N-terminal fragments of FMRP (NTDs). We did not observe an interaction between annealed BC1 RNA and FMRP_{1–280}, a construct comprising both N-terminal residues and the first KH domain (KH1). The results were consistent with previous work showing that this construct did not interact with unannealed BC1 RNA [41]. However, as FMRP_{1–280} did not interact with other nucleic acids under these conditions, we could not rule out the possibility that the protein was mis-folded although previous physical evidence suggests this is not the case [50]. Therefore, we also examined the interaction between annealed BC1 RNA and FMRP_{1–204}, a construct that does interact with nucleic acids and is expected “to be at least partially folded and be monodisperse” under the conditions it was used [50]. Nevertheless, annealed BC1 RNA did not interact with this FMRP fragment either. Hence, the first 204 residues of FMRP, which includes two Tudor domains and an alpha helix that is essential for domain stability [76], are not required for the interaction of annealed BC1 RNA with FMRP. Using different conditions, Zalfa et al. have suggested that BC1 RNA binds to an NTD comprising amino acids 1–217 [40]. The additional 13 residues of this construct comprise part of another alpha helix, which stabilizes the folding of FMRP’s KH1 domain [50]. While it is possible that amino acids 205–217 may confer binding specificity to annealed BC1 RNA, Zalfa et al. showed that a construct comprising this alpha helix along with the KH1 domain (residues 205–280) did not interact with BC1 RNA [40]. However, additional studies using other constructs will be needed to fully address the question of which FMRP residues are necessary and sufficient for its interaction with annealed BC1 RNA.

Although we demonstrate an in vitro interaction between FMRP and BC1 RNA, its nature and its physiological significance remain elusive. For example, the structure of the unique BC1 RNA conformer that FMRP binds to has not been defined other than the fact that it contains double-stranded RNA and is primarily found in the 5’ half of the molecule. In fact, it is possible based on the rather high RNA concentrations used in the annealing reaction that FMRP may be interacting with a dimer of BC1 RNA. This would be analogous to the formation of intermolecular G-quartet RNAs such as MAP1B RNA [69]. Clearly, a more comprehensive biophysical analysis of the parameters of this interaction is needed to fully address this issue. Regardless, the ultimate significance of this observation is questionable given that the formation of this conformer does not occur at temperatures that mammalian cells can survive. Although one might postulate that a chaperone protein may be able to mitigate the temperature requirement for annealing [77, 78], it is clear that FMRP cannot be this chaperone, because it is unable convert unannealed BC1 RNA into a molecule that it binds under physiological conditions. Given this, our data generated using different constructs, different preparations and different methods, converge with the published work of Iacoangeli et al. [47] to support a model in which FMRP and BC1 RNA operate independently of each other to control protein synthesis in neuronal processes.

Abbreviations

AGESA: Agarose electrophoretic mobility shift assays
BC1 RNA: Brain cytoplasmic RNA
eEF-1A: Eucaryotic elongation factor 1A
eIF4A: Eucaryotic initiation factor 4A
EMSA: Electrophoretic mobility shift assays
FMRP: Fragile X mental retardation protein
NTD: N-terminal domain
PABP: Polyadenylation binding protein
RBP: RNA binding protein
RRL: Rabbit reticulocyte lysate

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References


Review Article

Use of Specific Chemical Reagents for Detection of Modified Nucleotides in RNA

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Naturally occurring cellular RNAs contain an impressive number of chemically distinct modified residues which appear posttranscriptionally, as a result of specific action of the corresponding RNA modification enzymes. Over 100 different chemical modifications have been identified and characterized up to now. Identification of the chemical nature and exact position of these modifications is typically based on 2D-TLC analysis of nucleotide digests, on HPLC coupled with mass spectrometry, or on the use of primer extension by reverse transcriptase. However, many modified nucleotides are silent in reverse transcription, since the presence of additional chemical groups frequently does not change base-pairing properties. In this paper, we give a summary of various chemical approaches exploiting the specific reactivity of modified nucleotides in RNA for their detection.

1. Introduction

Native cellular RNAs contain numerous modified residues resulting from specific action of various RNA modification enzymes. These RNA modifications are ubiquitous in nature, but the specific modification profile varies depending on the organism. Over 100 chemically distinct modified nucleotides have been identified so far mostly in tRNAs, rRNAs, snRNAs and some snoRNAs. From the chemical point of view, these modifications are highly diverse and almost any position of the nucleobases as well as the 2′-OH of the ribose has been found to be a target of modification enzymes (see Table 1 and below) [1–3].

Identification of the chemical nature and localization of the modified nucleotides even in highly abundant RNAs represents a laborious and time-consuming task. Moreover, the analysis of low abundant cellular RNAs is extremely difficult due to limited access to highly purified RNA species required for most types of analysis, like HPLC or mass spectrometry [4–7]. One alternative to this consists in direct analysis of underrepresented RNA species in total cellular RNA by reverse transcription (RT) using specific DNA primers [8]. This generally allows the sequencing of a given RNA, but the information on its modified nucleotide content is still missing. The use of specific chemical reagents reviewed in this survey explores the particular reactivity of a given modified residue and may considerably help in the interpretation of an RT profile. Another area for the use of specific chemical reactions is RNA analysis and sequencing by various types of mass spectrometry (MS), where modified residues undergo particular fragmentation pathways and their derivatization helps in identification.

2. General Reactivity of Unmodified Nucleotides

Detection of nucleotide modifications is based on either of three basic principles. For two of these, that is, separation according to physicochemical properties and differential enzymatic turnover, we refer to other reviews [4, 7, 9–12] and will mention such methods only when they are combined with the use of chemicals that specifically react with modified nucleotides. Here, we focus on the third principle, which is differential chemical reactivity. To set the
Table 1: Known RNA modifications and their abbreviations and symbols (modified nucleosides mentioned in the text are indicated in bold).

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<tr>
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</tr>
<tr>
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<td>3-(3-amino-3-carboxypropyl)uridine</td>
</tr>
<tr>
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<td>ho'U</td>
<td>5-hydroxyuridine</td>
</tr>
<tr>
<td>63</td>
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</tr>
<tr>
<td>64</td>
<td>cmo'U</td>
<td>uridine 5-oxoacetic acid</td>
</tr>
<tr>
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</tr>
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<tr>
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stage, we will outline the known reactivity of the four major ribonucleotides in this paragraph. In principle, any reagent that reacts with nucleotides may be considered for chemical recognition of nucleotide modification, provided that conditions can be determined, where its reactivity significantly differs between a given major nucleotide and its modified counterpart. In the best case, conditions would be optimized to the point of exclusive reaction with either the standard or the modification. From a practical point of view, it is helpful to distinguish reagents within a narrowly defined window of experimental conditions from those which reliably and completely discriminate nucleotides over a wide plateau of experimental conditions. The former methods are evidently more difficult to newly establish in a laboratory, despite the emergence of numerous publications covering detailed protocols, and should be tackled only by experienced RNA scientists. However, even for the less complicated reactions, one should be conscious that deviations in temperature, pH, salt, incubation time, or reagent concentration may result in “leaving” the plateau and thus result in suboptimal discrimination. Similar considerations apply to the detection of the chemical species resulting for treatment with the discriminating reagent. Most often, the ease and turnaround time of detection will determine if a given reagent finds widespread use in the RNA community.

The above considerations apply to reagents of various types alike, which we have grouped into electrophiles, nucleophiles, and oxidizing, and reducing agents.

2.1. Electrophilic Reagents. Nitrogen atoms in purine and pyrimidine rings of nucleobases show varying degrees of nucleophilicity and thus react differentially with various electrophilic compounds, which are typically alkylating or acylating agents (Figure 1).

These reactivities have been explored decades ago and form the basis of chemical sequencing of end-labeled nucleic acids according to Maxam and Gilbert [13–15]. Methylating agents, in particular dimethylsulfate (DMS), preferentially alkylate the N1 of adenosines, the N7 of guanines, and the N3 of cytidines. All three sites are also found to be enzymatically methylated in vivo, and the methods used for sequencing of chemical probing of RNA structure can also be applied to reveal these naturally occurring modifications. An important property of many modified nucleotides is that they render oligonucleotides susceptible to chain scission via β-elimination, provided that the modification itself or an auxiliary chemical treatment ablates aromaticity of the base or leads to abasic sites. β-elimination is induced by heating of RNA with aniline at low pH. Additional treatment of m6C with hydrazine and of m7G [16], dihydrouridine, and wybutosine with sodium borohydride (NaBH4) leads to abasic sites in RNA which are susceptible to aniline cleavage. The wybutosine base is also acid-labile and will undergo depurination upon treatment with hydrochloric acid to leave an abasic site [17]. To date, there is no satisfactory protocol to chemically reveal the presence of N1-methyladenosine in RNA, which therefore has to be detected by RT-based methods, as will be detailed below.

Other popular electrophilic agents that react with nitrogens include diethyl pyrocarbonate (DEPC, N7 of adenosine) and ethylnitrosourea, which alkylates all nitrogens in addition to phosphates and all oxygens in nucleic acids [18]. N,N-(dimethylamino) dimethylchlorosilane (DMAS-Cl) reacts with the N2 of guanosines [19]. Certain reagents gain in specificity due to the presence of two electrophilic groups, for example, chloroacetaldehyde (N1-A, N6-A N3-C, N4-C) [20, 21], chlorotetrolic (4-chloro-2-butynoic) acid ester (N1-A, N6-A N3-C, N4-C) [22], and glyoxal and kethoxal (N1-G, N2-G) [23, 24]. Finally, carbodiimides, in particular 1-cyclohexyl-3-(2-(4-morpholino)ethyl) carbodiimide tosylate (CMCT), acylate the N3 of uridines and the N1 and N2 of guanosines, as well as both nitrogens in pseudouridine and N3 in inosine. Its use for the mapping of Ψ residues will be detailed below.

2.2. Oxidizing Agents. As electron poor species, oxidizing agents share certain properties and also target moieties with alkylating agents. Nitrosyl cations and related species react with exocyclic nitrogens in cytidine, adenosine and guanosine to form diazonium compounds, which eventually hydrolyze and yield the corresponding deamination products: uridine, inosine, and xanthene, respectively. Monopersulfonic acid was reported to oxidize adenosine to yield adenosine 1-oxide [25]. The A5,6 double bonds in pyrimidines can in principle be oxidized by hydrogen peroxide although permanganate and in particular osmium-VIII compounds are most frequently used (reviewed in [26]).
Radical generating species, including Fenton reagents [27], copper phenantroline [28, 29] and peroxonitrous acid [30] are known to abstract hydrogens from CH bonds in the ribose. So far, this is the only type of reactivity that has not been exploited for the detection of nucleotide modifications. This type of reagents also causes the formation of 8-oxoguanosine, which is the major lesion in oxidative damage of DNA [31].

2.3. Nucleophilic and Reducing Reagents. In pyrimidines, the Δ5,6 double bond is part of a Michael acceptor, which can be attacked in the 6 position by strong nucleophiles such as, bisulfite, hydrazine, methoxamine, hydroxylamine [32–34], and bisulfite [35–37] but also by KI/TiCl3 [38]. NaBH4 will act only on methylated N3-C, as pointed out already.

The C4 position is also electrophilic, but typically less reactive than the Michael acceptor electrophile C6. Nucleophilic attack here is known from the bidentate hydrazine [39], or a combination of bisulfate and a semicarbazide [40]. In both cases, the first nucleophilic attack occurs at the C6, and only then does the C4 position react [33, 39]. Using hydrogen sulfide under high pressure, cytidines can be converted into s4U as the result of a nucleophilic attack at the carbon 4 position [41, 42], which might be preceded by an initial nucleophilic attack at the C6.

3. Specific Reactivity of Modified Nucleotides

From a perspective of chemical reactivity, it is helpful to distinguish between modifications carrying principally new chemical moieties, and those which only moderately alter the reactivity of chemical moieties already present in RNA. The latter category essentially contains all methyl group additions, and reagents and reaction conditions must be carefully controlled to achieve discrimination. Thus, the detection of m3C, which has already been mentioned (see Figure 2(a)), is based on the susceptibility of cytidines towards nucleophiles, which was increased by the methyl group modification to N3, and thus allowed for detection after hydrazine treatment. In contrast, addition to the 5 position of a methyl group renders cytidines more electron rich and consequently less electrophilic. Therefore, nucleophilic attacks by bisulfite can be restricted to cytidines under carefully controlled conditions which leave m5C unaffected by bisulfite.

A number of more sophisticated modifications involve the introduction of chemical moieties which are chemically orthogonal, that is, they can be conjugated by certain reagents to which unmodified RNA is completely inert. Thus, isothiocyanate and NHS-derivatives selectively react with primary amines as present, for example, in acp3U, or lysidine (k 2C). Thiolated nucleotides such as s 2U, s 4U, and s2C [43, 44] react with iodoacetamide derivatives, and carboxylic acids such as in t 6A/m6t6A and acp3U [45, 46], other COOH-containing nucleotides can most probably be activated by carbodiimides for conjugation with a nitrogen nucleophile.

3.1. 5-Methylcytosine. The chemistry of m5C in DNA has been subject to intense research as a consequence of the
importance of m5C in the field of epigenetics. Bisulfite m5C sequencing is a state-of-the art detection method and used on a very large scale. The addition of bisulfite to the 6 position promotes a series of chemical transformations depicted in Figure 2(b), which ultimately leads to deamination. Cytidines are thus transformed into uridines and read as such in subsequent sequencing reactions. Based on their resistance to deamination reaction, sequencing after bisulfite treatment will reveal m3C residues as cytidine signals. Despite lasting efforts, adaptation of bisulfite to RNA has only recently succeeded [35–37] and is now one of the few methods that might be used to investigate RNA modification on a genome-wide scale [47, 48].

As indicated in Figure 2(c), another avenue of chemical discrimination between cytidines and m3C may involve oxidation of the double bond. Although the oxidation with permanganate produces insoluble MnO2, and further oxidation of the glycol to bis-aldehyde may occur under certain conditions, it has been successfully employed in the discrimination of deoxy-m3C versus deoxycytidine [49, 50]. The osmium tetroxide reactions do not involve the formation of insoluble products, but the reagent is highly toxic and mutagenic. In the past years, a variety of osmate-based bioconjugate reagents have been developed for the selective detection of m3C in DNA. These reagents exploit the additional electron density brought about by the methyl group for highly selective formation of a stable complex with deoxy-m3C but not with deoxycytidine. However, applications to RNA are still lacking [51–53] (reviewed in [26]).

3.2. 3-Methylcytosine. Detection of m3C is based on its increased reactivity towards hydrazine, as discussed above. Figure 2(a) shows the reaction sequence leading to an intermediate susceptible to aniline-induced cleavage via β-elimination. Cleavage sites can be revealed by sequencing reactions with end-labeled RNA [14].

3.3. 7-Methylguanosine. Guanosine methylated at position 7 (m7G) is frequently present in the variable region of tRNAs. As has been pointed out, its detection can be achieved by aniline-induced cleavage of the RNA chain by β-elimination after additional treatment under alkaline conditions [16] or after its reduction by sodium borohydride (NaBH4, shown in Figure 3). Because solutions of NaBH4 are typically alkaline, and because the reduction product has been described as sensitive to reoxidation in air, the actual species undergoing β-elimination may not be well defined. This reaction was first studied for isolation of defined tRNA fragments [54] and later applied to RNA sequencing using DMS in Maxam and Gilbert type chemistry [14] or RNA structural probing.

3.4. 2'-O-Methylated Riboses. Alkaline hydrolysis of RNA polynucleotide chains proceeds via deprotonation of the ribose 2'-OH to the corresponding alcoholate, and its nucleophilic attack of the nearby 3'-phosphate. The resulting intermediate is unstable and rapidly decomposes into a 2',3'-cyclophosphate leading to phosphodiester bond cleavage (Figure 4(a)). The methylation of ribose 2'-OH prevents alcoholate formation, and thus decreases the reactivity of the 2'-oxygen and consequently prevents phosphodiester bond cleavage almost completely [55, 56]. This resistance to alkaline hydrolysis of the phosphodiester bonds on the 3’-side of 2'-O-methylated nucleotides can be detected by primer extension or by direct analysis of the cleavage profile of end-labeled RNA. The 2'-O-methylated residue appears as a “gap” in the regular ladder of OH- cleavage.

A very sensitive method involving RNase H digestion directed by 2'-O-methyl RNA-DNA chimeras can be used to confirm the presence of a 2'-O-methylated residue. RNase H nonspecifically cleaves the RNA strand of an RNA-DNA hybrid. However, it does not cleave any site where the 2'-O position of an RNA residue is methylated. The digestion of an RNA directed by a complementary 2'-O-methyl RNA-DNA chimeric oligonucleotide consisting of four deoxynucleotides flanked by 2'-O-methyl ribonucleotides is site specific. The use of a 2'-O-methyl RNA-DNA chimera and RNase H digestion therefore provide a direct assay for determining whether any particular nucleotide in a long RNA molecule carries a 2'-O-methyl group. If the 2'-O position of the targeted residue is methylated, RNase H cleavage is blocked. If instead the 2'-O position is unmodified, RNase H should cut the RNA specifically at that site [57].

Another method based on the RNA treatment by sodium periodate followed by β-elimination has been recently used to analyze the methylation status of the 3’-terminal nucleotide of plant miRNAs [58, 59]. The reactions eliminate a 3’ nucleotide containing both 2'- and 3'-OH groups on the ribose (Figure 4(b)), resulting in an RNA product that is one nucleotide shorter than the substrate RNA and that contains a 3’ phosphate group [60]. Therefore, miRNAs that contain a 3’ terminal ribose with both 2’- and 3’-OH groups will migrate faster during electrophoresis between one and two nucleotides after the treatment. Methylated miRNAs whose 3’ terminal ribose contains a 2'-O-methyl group do not participate in the reactions and remain unchanged in mobility. A significant disadvantage of this approach is that it does not distinguish a 2'-O-methyl modification from other potential modifications on the 3’ terminal ribose. In studies by Yu et al., it was possible to assign the identity of the 3’-terminal nucleotide modification thanks to mass spectrometry studies [58].

More recently, an alternative chemical treatment which should allow the distinction between unmethylated and 2'-O-methylated residues has been proposed [61, 62]. Free unconstrained ribose 2’-OH easily reacts with N-methylisatoic anhydride (NMIA, Figure 4(c)), and the resulting adduct can be analyzed by primer extension (selective 2’-hydroxyl acylation analyzed by primer extension, SHAPE). For the moment, the method was only applied to RNA structural analysis. However, comparisons of NMIA modification and primer extension profiles for unmodified transcript and 2'-O-methyl-containing RNA may probably be used as a tool for 2’-O-methylation mapping.

Yet another approach allowing the separation of O-methyl nucleosides from ribose-unsubstituted nucleosides in one chromatographic step has been developed in the early 80s [63]. This methods is based on the capability of boronate
Figure 2: Specific chemical reactions for \( m^3 \text{C} \) and \( m^5 \text{C} \) detection. (a) Cleavage of \( m^3 \text{C} \) and unmodified uridine by hydrazine. (b) Deamination of unmodified cytosine by bisulfate; \( m^5 \text{C} \) is resistant for deamination. (c) Specific oxydation of 5-6 double C=C bond in methylated pyrimidines by \( \text{MnO}_4^- \), \( \text{OsO}_4 \), and \( \text{OsO}_4 \) derivatives.
Figure 3: Sodium borohydride (NaBH₄) reduction of m⁷G in RNA. Reduction of m⁷G leads to formation of an abasic site in RNA followed by the cleavage of the RNA chain by β-elimination.

to form an anionic complex with the cis-2',3'-hydroxyls of unsubstituted ribonucleosides, but not with ribose-substituted nucleosides such as 2'-O-methylribonucleosides. Phenyl boronates with hydrophobic side chains of about 1 nm in length have been synthesized and used to coat inert 10 μm beads of polychlorotrifluoroethylene. This matrix complexes easily with compounds containing free vicinal cis-hydroxyls allowing their separation from their O-alkyl or O-acyl derivatives. Boronates also find application in affinity electrophoresis [64], as will be detailed later on.

3.5. Pseudouridine. Pseudouridine (psi, Ψ) is the result of an isomerization reaction during which the C5 and N1 positions of uracil are interconverted. The resulting nucleotide features a glycosidic carbon-carbon bond and an additional amide functionality, which is available for chemical discrimination (Figure 5). Because Ψ retains the base pairing characteristics of uridine, it is invisible in RT profiles or by RT [8]. Somewhat surprisingly, chemical modification can be achieved with satisfying selectivity by two classes of electrophiles. Michael acceptors such as acrylonitrile [68] or methylvinylsulfone [69] alkylate the N3 while not reacting with the four major nucleotides (Figure 5(b)). These reagents are, however, known to react also with inosine, m⁵C, and m⁵U [69, 70].

Ψ residues can be acylated on both nitrogens by carbodiimides (Figure 5(a)) [71, 72]. The reaction also acylates both the N3 of uridines and the N1 of guanosines, but all residues except for the one on N1 of Ψ can be removed by subsequent alkaline treatment [73, 74]. Indeed, whereas U and G adducts of CMC are readily cleaved by weakly alkaline conditions, Ψ residues, being resistant to hydrazinolysis, are not cleaved and do not stop RT.

3.6. Thiolated Nucleotides. Sulfur atoms, even when introduced into RNA as thiocarbonyl functions, are easily oxidized but are also very nucleophilic and thus react with a variety of electrophiles under conditions under which the remainder of RNA residues stays inert. Iodoacetamide derivatives, for example, of fluorescent dyes, are typical reagents to derivatize sulfur residues in proteins, that is, free cysteins. Diluted solutions of these reagents have been shown to selectively conjugate to thiolated nucleotides such as s²U, s⁴U, and s²C [43, 44] (Figure 6). Thiouridines have also been reported to react with carbodiimides like CMCT [78], and the resulting conjugates can be identified by mass spectroscopy [65, 67].

The thiophilic character of mercury ions has been exploited for affinity electrophoresis in polyacrylamide gels copolymerized with (N-acryloylamino)phenyl-4-mercuric chloride (APM-gels, Figure 6). Sulfur-containing RNAs are retarded during migration, and interestingly, the degree of retardation depends on the nature of the thiomodification; s⁴U is more strongly retarded comparing to s²U [79].

3.7. Modifications Containing Primary Amines. Primary amines, which are not present in unmodified RNA, are relatively strong nucleophiles which can be selectively conjugated to a number of commonplace amino-derivatizing agents
containing, for example, $N$-hydroxysuccinimide and isothiocyanate moieties [78, 80, 81], and probably with pentafluorophenol derivatives, too (Figure 7(a)). For example, labeling with fluorescent dyes has been successfully implemented for E. coli tRNA$^{Phe}$ containing acp$^3$U [78, 81]. Fluoresceinisothiocyanate under mild alkaline conditions was successfully applied for the labeling of E. coli tRNA$^{Tyr}$ at the queosine position [82]. Free amino functions may also react with
3.8. Modifications Containing free Carboxylic Acid Groups.
A number of modifications typically occurring at positions 20, 34, and 37 of tRNAs consist in the incorporation of an amino acid via its amino function, resulting in the presence in the modified RNA of a free carboxy group. This can be selectively activated for conjugation with amine reagents by dilute water-soluble carbodiimide, which will not react with the other nucleobases under these conditions (Figure 7(b)). The principle has been demonstrated with dansyl chloride under neutral conditions, as was demonstrated by selective labeling of preQ_{1-34} in the same *E. coli* tRNA^{Tyr} obtained from queosine-deficient strain [83] (Figure 7(a)).
Figure 7: Specific reactivity of free NH₂– (a) and –COOH (b) groups in modified nucleotides. acp³U is taken to illustrate the reactivity with isothiocyanate derivatives and NHS derivatives. Reaction with dansyl chloride is shown for preQ₁ (a). Reactions of free –COOH groups with aniline, ethylenediamine and similar molecules in the presence of soluble carbodiimide. The resulting free NH₂-group of ethylenediamine may be further used for attachment of activated acyl (b). The structure of mt⁶A(m₆t⁶A) is shown at the bottom.
3.9. Dihydrouridine. In contrast to all other pyrimidines, the dihydrouridine (D) has a saturated pyrimidine ring due to reduction of the double C\(=\)C bond between carbons 5 and 6. This considerably affects the stability of D both in the reduction by NaBH\(_4\) [84–86] and under alkaline conditions. In both cases, cleavage of the dihydrouridine ring is followed by cleavage of the RNA chain.

mt\(^6\)A (mt\(^6\)t\(^6\)A) and acp\(^3\)U [45, 46] but may be applicable to a variety of other modifications, potentially including ms\(^2\)t\(^6\)A, ms\(^4\)t\(^6\)A, g\(^6\)A, hn\(^6\)A, and ms\(^2\)hn\(^6\)A, chm\(^5\)U, cmo\(^5\)U, cmnm\(^5\)s\(^2\)U, cmnm\(^5\)Um, cmnm\(^5\)Um, m\(^1\)acp\(^3\)Ψ, τm\(^5\)s\(^2\)U, and possibly others.
conditions [87]. Initial studies of tRNA nucleosides by NaBH₄ treatment revealed that D, ac⁴C, and s⁴U are reduced under rather harsh reaction conditions [84], while major nucleosides, as well as Y residues, were found to be inert. The product of D reduction by NaBH₄ is a ureidopropanol riboside (Figure 8(a)). Further studies demonstrated that the D residue in tRNA can be selectively reduced by NaBH₄ under very mild conditions at pH 7.5 in Tris-HCl buffer on ice [86].

Another characteristic of D is its particular sensitivity to alkaline hydrolysis. Hydrolytic ring opening (Figure 8(b)) of dihydrouridine occurs quite rapidly at elevated temperatures and a pH above 8.0–8.5 [88]. This instability may explain the absence of this modification in hyperthermophiles and was also used for detection of D residues in yeast tRNA and characterization of the corresponding tRNA D-syntases [87].

3.10. Inosine. The modified nucleotide inosine (I) is derived from adenosine (A) by enzymatic deamination catalyzed by specific adenosine deaminases [89, 90]. RNase T₁ does not distinguish inosine residues present in RNA from guanosine residues and cleaves on their 3’ side. However, preliminary specific reaction with glyoxal allows RNase T₁ to distinguish G and I residues [91] (Figure 9(a)). Indeed, glyoxal reacts with N₁ and N₂ of G residues and thus abolishes their recognition by RNase T₁. The resulting covalent adduct is stabilized by boric acid. By contrast, inosine cannot react in the same way with glyoxal due to the absence of NH₂-group at position 2 and the glyoxal-inosine adduct is unstable. Thus, RNase T₁ cleavage of glyoxal-treated RNA proceeds only at inosine residues and not at G residues. The cleavage positions may be detected by primer extension analysis or other methods.

3.11. Queuosine. The complicated hypermodification queuosine contains two vicinal hydroxyl groups, a feature otherwise only present in the most 3’-ribose of an RNA chain. A chelating effect of these hydroxyl groups can be exploited by complexation to Lewis acids such as boronic acid. Igloi and Kössel [64] have exploited this feature to develop affinity electrophoresis based on the incorporation of (N-acryloylamino)phenyl-3-boronic acid (APB) into polyacrylamide gels (Figure 9(b)). Affinity chromatography based on the same principle has been described by Vogeli et al. [92]. Of note, vicinal hydroxyl groups can be selectively oxidized to yield dialdehydes which can be selectively condensed with hydrazine derivatives, yielding hydrazones that can be further stabilized by reduction with borohydrides.

3.12. Wybutosine. The reaction of the wybutosine base (yW) in yeast tRNA^{Phe} with NaBH₄ has already been mentioned. Its depurination under mild HCl treatment (pH 2.9, 37°C, 2-3 hours, Figure 10) was already noticed in the late 60s and 70s [17, 93]. Later on, the reaction of the riboaldehyde group formed at the resulting abasic site with hydrazine and hydrazine derivatives was used for specific fluorescence labeling of tRNA^{Phe} for biophysical studies of the ribosomal translation [86, 94].

4. Practical Use of Specific Reagents for Detection of Modified Nucleotides by RT or RNA Chain Cleavage

Specific chemicals like those described above may be used both for detection and the precise localization of modified residues in RNAs. The techniques developed for RNA sequencing using Maxam and Gilbert’s approach also allow for detection of modified nucleotides, since many chemical reactions that are specific for modified nucleotides may be exploited to induce cleavage of RNA polynucleotide chain, often in combination with further chemical treatment. This is the case, for instance, for hydrazine cleavage, NaBH₄ reduction, or hydrolysis of phosphodiester bonds under
various conditions. Analysis of the cleavage position can be performed with 5'-32P-labeled RNA and separation of the cleaved fragments by electrophoresis on denaturing PAGE. However, these techniques require difficult and time-consuming purification to homogeneity of a sufficient amount (at least 0.5–1 μg) of a given RNA species and thus cannot be applied to low-abundance cellular RNAs.

An alternative approach for the detection of RNA modifications is based on the use of RT. A synthetic DNA oligonucleotide designed to target a specific sequence is annealed to cellular RNA without previous RNA purification. Primer elongation by reverse transcriptase allows both sequencing of RNA species by Sanger type reactions and, in addition, the detection of specific reagent-dependent stops, which are indicative of the presence and exact position of modified nucleotides. A limited number of natural RNA modifications altering Watson-Crick base pairing can be detected by this approach even in the absence of a preceding chemical treatment. For RT-silent modifications, a specific reagent is required to create a bulky chemical modification, which blocks base pairing and consequently progression of the reverse transcriptase, thus resulting in an RT stop. Alternatively, cleavages of the RNA chain generated by specific treatment may also be detected by primer extension. However, interpretation of RT profiles should be undertaken with caution, since the presence of stops or gaps in the RT profile is frequently hidden by natural pauses of the RT at the corresponding positions.

For some RNA modifications, a simple primer extension under specific conditions may also be used for detection. Such a method was developed for the precise mapping of 2'-O-methylated residues in RNA and uses primer extension by reverse transcriptase at low dNTP concentrations [55, 56]. It is based on the observation that limited dNTP concentrations cause the reverse transcriptase to frequently slow down (or pause) at such nucleotides. Primer extension with normal (unmodified) residues is much less sensitive to reduced dNTP concentration. Many other modified nucleotides in RNA do not create such pauses, and thus low dNTP concentration-dependent stops seem to be rather specific to 2'-O-methylation. The comparison of RT profile for natural (modified) RNA and unmodified RNA transcript may help to discriminate modification-dependent from other RT stops. It is noteworthy that pauses observed at 2'-O-methylated residues are sequence dependent and, depending on the sequence context, no pause may occur at some of the 2'-O-methylated residues. Therefore, the method does not allow an exhaustive identification of all 2'-O-methylated residues in RNA molecules. In some instances, RNA sequence changes resulting from RNA editing (e.g., adenosine or cytidine deamination) can also be directly detected by comparison of RT profile for unmodified transcript and natural RNA species.

Other methods employing RT are based on particular base pairing properties of some modified nucleotides. An alternative approach for inosine detection consists in direct RNA sequencing with RT and comparison of the cDNA sequence obtained with the corresponding genomic sequence. Due to the absence of amino group at position 6, I base pairs with C residues instead of U residues, thus change the sequence of the cDNA synthesized by RT. Thus, one can detect unexpected A → G changes in the cDNA compared to the genomic sequence [95]. To confirm the data, it is also possible to compare the cDNA sequence obtained by direct sequencing of an unmodified transcript produced by in vitro transcription with the cDNA sequence obtained with the authentic RNA. Another recently developed method that is suitable for transcriptome-wide analysis of inosine is based on specific cyanomethylation of inosine [70].

5. Conclusion

Despite extensive development of specific chemical reagents during several decades (starting in the 1970s), the spectrum of available chemicals capable of selective reaction with modified ribonucleotides remains rather limited. This is related to a limited difference of chemical reactivity between modified nucleotides and their unmodified counterparts. Furthermore, the low stability of RNA molecules during required reaction conditions presents a stringent limitation. Due to these limitations, development of specific reaction schemes was possible only for a small subset of the known modified residues in RNA. Future development in the field should fill these gaps and propose new chemical reagents for extensive analysis of RNA modification by RT and by MS techniques.

Abbreviations

APM: (N-acryloylamino)phenyl-4-mercuric chloride
APB: (N-acryloylamino)phenyl-3-boronic acid
RT: Reverse transcription
TLC: Thin layer chromatography
CMCT: 1-cyclohexyl-3-(2-((4-morpholiny1)ethyl)carbodiimide)tosylate
DMS: Dimethylsulfate
DEPC: Diethyl pyrocarbonate
DMAS-Cl: N,N-((dimethylamino)dimethylchlorosilane
NHS: N-hydroxsuccinimide.

References


Research Article

Branched RNA: A New Architecture for RNA Interference

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Branched RNAs with two and four strands were synthesized. These structures were used to obtain branched siRNA. The branched siRNA duplexes had similar inhibitory capacity as those of unmodified siRNA duplexes, as deduced from gene silencing experiments of the TNF-α protein. Branched RNAs are considered novel structures for siRNA technology, and they provide an innovative tool for specific gene inhibition. As the method described here is compatible with most RNA modifications described to date, these compounds may be further functionalized to obtain more potent siRNA derivatives and can be attached to suitable delivery systems.

1. Introduction

In recent years, siRNAs have generated tremendous interest in therapeutics [1]. Nevertheless, the transition of siRNAs from the laboratory to the clinical practice has encountered several obstacles. Briefly, siRNA duplexes are rapidly degraded in serum by exonucleases and endonucleases [2]. The polyanionic phosphodiester backbone of siRNA suffers from difficult cell uptake [3], and oligonucleotides may have off-target effects, either by stimulating the immune system [4] or by entering other endogenous gene regulation pathways [5]. Several chemical modifications have been proposed in the literature to address these drawbacks [2–4]. Most of these modifications are based on modified nucleosides and changes on backbone linkages [6, 7]. Thus, changes in sugar moiety influences sugar conformation, and, therefore, overall siRNA structure. Modifications of the 2′-OH by F or OMe as well as LNA [8, 9] are well tolerated and improve binding affinity and nuclease resistance. Base modifications that stabilize base pairs (5-bromouracil, 5-methylcytosine, 5-propynyluracil, and others) have also been proposed [7, 10]. Terminal conjugates, especially at the termini of the sense strand, have been modified with a large number of lipids to achieve improved cellular uptake [11].

In addition to these modifications, siRNA architecture is also crucial in the design of effective and specific siRNA. The architecture itself can be altered by chemical synthesis. In addition to the canonical siRNA architecture of 21-nt antiparallel, double-strand RNA with 2-nt 3′-overhangs [12], several forms of siRNA have been described. Blunt-ended siRNA [13], 25/27 mer Dicer-substrate or asymmetric siRNA [14] are among the siRNA structures formed by two strands. Moreover, functional siRNA can also be formed by one single RNA strand. This is the case in small hairpin RNA (shRNA), where the two strands are linked by a single loop [15], or RNA dumbbells [16], made by closing the open end of the hairpin. This last structure retains RNAi activity while providing complete protection from nucleases [16]. Finally, siRNA can also comprise three strands, namely, two 9–13 nt sense strands and the intact antisense strand. This structure is known as small internally segmented interfering RNA [17] (sisiRNA). Some of these modifications have reduced off-target effects and increased potency (Figure 1(a)). Another architecture not yet explored in siRNA is the branched RNA structure obtained from a central building unit and several branching points that enable the strand growth.

Several strategies can be used to prepare branched RNA structures. Although the synthesis of these compounds is
complex and tedious, commercially available synthons have improved the complexity and yields of these structures.

The assembly of branched nucleic acids on a solid support can be achieved by convergent or divergent strategies. In the former, synthesis of branched oligonucleotides containing two or more identical strands can be achieved by branching derivatives 1,3-diaminopropanol, pentaerythritol, the commercially available symmetric doubler [18–20], or by a ribonucleoside bisphosphoramidite [21] as synthons. In contrast, in the divergent approach two or more distinct strands are prepared with synthons with orthogonal support can be achieved by convergent or divergent strategies.

2. Experimental Section

2.1. Oligonucleotides. The following RNA sequences were obtained from commercial sources (Sigma-Proligo, Dharmacon): sense or passenger scrambled 5′-CAGUCGCGUG-UUGGAGCACUGG-dT-dT-3′, antisense or guide scrambled 5′-CCAGUGCAAAAGCCACUG-dT-dT-3′, antisense or guide anti-TNF-α: 5′-GAGCUGAGAUAAGAC-dT-dT-3′, and sense or passenger anti-TNF-α: 5′-GUGCCUU-AUGUCUCAGCCUC-dT-dT-3′. RNA monomers in capital letters, dT represents thymidine. The anti-TNFα siRNA was previously described to efficiently downregulate murine TNFa mRNA [36].

Figure 2 refers to the branched RNA structures synthesized in this study. DB stands for the symmetric doubler phosphoramidite obtained from commercial sources (Glen Research). Guanosine was protected with the dimethylaminomethylidene group, cytidine with the acetyl group, and adenosine with the benzoyl group. t-Butyldimethylsilyl (TBDMS) group was used for the protection of the 2′-OH function of the RNA monomers. The phosphoramidites were dissolved in dry acetonitrile (0.1 M), and a modified cycle was used with increased coupling time to 10 min. Oligoribonucleotide 1 was synthesized on a CPG solid support with a symmetric branching unit of two arms containing two DMT-protected hydroxyl groups, as described in [31]. Oligoribonucleotides 2 and 3 were synthesized using standard low-volume polystyrene thymidine columns. After the solid-phase synthesis, the supports were treated with concentrated aqueous ammonia-ethanol (3:1) for 1 h at 55°C. After filtration of the supports, the solutions were evaporated to dryness. The residue was dissolved in 85 µL of 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) for 12 h. Then, 85 µL of 1 M of triethylammonium acetate was added and the oligoribonucleotides were desalted on a NAP-10 column using water as eluent. The compounds were purified by HPLC under the following conditions. Column: Nucleosil 120–10 C18 (250 × 4 mm); 20-min linear gradient from 15% to 100% B (DMT ON conditions); flow rate 3 mL/min; solution A was 5% acetonitrile in 0.1 M aqueous triethylammonium fluoride (TEAA) buffer and B 70% acetonitrile in 0.1 M aqueous TEAA. The purified products were analyzed by MALDI-TOF mass spectrometry. Yields (0.2 µmol scale synthesis) were between 5–10 OD units at 260 nm.

2.2. Thermal Denaturation Studies. The thermal melting curves for duplexes of the oligoribonucleotides 1–3 and their unmodified RNA complementary strands (guide strand) were performed following the absorption change at 260 nm. Samples were heated from 20°C to 80°C, with a linear temperature ramp of 0.5°C/min in a JASCO V-650 spectrophotometer equipped with a Peltier temperature control. Sample concentration of the samples was around 2 µM. All the
measurements were repeated three times and conducted in 15 mM HEPES 1 mM Mg(OAc)₂ and 50 mM KOAc pH 7.4.

2.3. Cell Culture, Transfection, and Cellular Assays. HeLa cells were cultured under standard conditions (37°C, 5% CO₂, Dulbecco’s Modified Eagle Medium, 10% fetal bovine serum, 2 mM L-glutamine, supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL)). All in vitro experiments were performed at 40–60% confluence. HeLa cells were transfected with 250 ng of a plasmid expressing murine TNF-α using lipofectin (Invitrogen), following the manufacturer’s instructions. One hour after transfection cells were transfected with 100 nM double strand concentration of siRNA against TNF-α, using oligofectamine (Invitrogen).

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Figure 2: Schematic representation of the chemical structure of the branching units of the oligonucleotides described in this study.
Previously, siRNA duplex annealing was performed by mixing modified (1, 2, 3) and unmodified passenger strands (unm) with the appropriate amount of the corresponding unmodified guide strand.

TNF-α concentration was determined from cell culture supernatant by enzyme-linked immunosorbent assay kit (Bender MedSystems) following the manufacturer’s instructions. The inhibitory capacity of the siRNA duplexes is expressed as double strand concentration for comparative purposes. A 100 mM double strand concentration is equivalent to a 50 nM concentration of two-branched siRNA (1 or 2) and to 25 nM of four-branched siRNA (3).

3. Results and Discussion

3.1. Oligonucleotide Synthesis. In order to prepare branched RNA for RNA interference, the potential steric hindrance of the branching unit with RISC must be considered. As the passenger strand is removed from the siRNA duplex upon binding to RISC, we introduced the branching modification at the protruding 3′-end of the sense strand. This position has been demonstrated to allow the introduction of a large number of modifications without affecting the inhibitory capacity of siRNA [6, 7]. We thus designed branched oligonucleotide sequences 1–3 of the passenger strand of a siRNA directed against TNF-α (Figure 2). Sequence 1 was synthesized using a controlled pore glass (CPG) solid support containing a symmetric doubler [31], as shown in Figure 3. Sequences 2 and 3 with two or four strands, respectively, were synthesized on a low-volume polystyrene support (LV200) functionalized with dimethoxytrityl- (DMT-) thymidine. The commercially available symmetric doubler phosphoramidite was used to introduce two and four branches on the 3′-position of the starting thymidine (Figure 3). Sequences were assembled using standard protocols for RNA synthesis. The 2′-OH function of ribonucleosides was protected with the t-butyldimethylsilyl (TBDMS) group. Coupling yields, determined by the absorbance of the DMT

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**Figure 3**: Outline of the synthesis of branched RNA oligonucleotides 1, 2, and 3 with two and four arms with identical sequence. (a) Assembly of the RNA sequence by standard solid-phase methods; (b) removal of protecting groups and release of the RNA molecule from solid support; (c) addition of the symmetrical branching phosphoramidite.

**Figure 4**: HPLC profile of DMT-containing oligonucleotide 2 with two arms. Truncated sequences without DMT groups had a retention of less than 5 min. Fraction eluting between 7-8 min contained oligonucleotides with a single DMT group. The last fraction contained the desired sequence with two DMT groups.
cation released in each synthesis step, were more efficient (98%) on low-volume (LV200) polystyrene supports than on CPG support (95%). After assembly of the sequences, the DMT-containing oligonucleotides were released from the supports with ammonia, and the resulting compounds were treated with fluoride to remove the TBDMS groups. HPLC analysis of the resulting products is shown in Figures 4 and 5. Several peaks were observed for the synthesis of the two-branch RNA sequences (1 and 2; Figure 4). Truncated sequences without DMT groups eluted between 3–5 min. A fraction containing oligonucleotides with a single DMT group was eluted next, and the last fraction contained the desired sequence with two DMT groups. Mass spectrometry analysis (Table 1) and electrophoresis analysis confirmed the mass and size of the desired branched oligoribonucleotides.

Figure 5 shows the HPLC profile of the mixture obtained in the synthesis of the four-branch RNA sequence (3). In this case, three peaks in the DMT-containing area were observed. Although resolution of these peaks was not as good as in the previous case, the last eluting peak corresponded to the desired tetra-DMT compound (3). The purified compound had the expected molecular weight, was homogeneous by analytical HPLC (Figure 5), and showed the correct migration in polyacrylamide gel electrophoresis (PAGE).

3.2. Thermal Denaturation Studies. The melting temperatures of the branched siRNA duplexes formed by annealing of equimolar amounts of sequences 1–3 with unmodified passenger strand are shown in Table 1. Duplex 1 had the lowest melting temperature, which was 3.5°C lower than the unmodified duplex (Table 1). Duplex 2 melted 1.5°C lower than the unmodified duplex. In contrast, duplex 3 had similar melting temperatures as the unmodified duplex. The small decrease in melting temperatures of the two-branched siRNA structures is possibly due to a steric effect in the branching point that holds the two duplex strands in close proximity. The four-stranded architecture had a larger separation between strands as a result of the introduction of 3 branching units, thus the resulting duplexes showed greater similarity to the unmodified duplex. Thus we believe that the small destabilizing effect observed in the two-branched RNA duplexes could be optimized in further experiments by adding a linker between the branching unit and the RNA strands, as described by Grimau et al. [31].

3.3. Cell Culture, Transfection, and Cellular Assays. Tumor necrosis factor (TNF-α) was selected as a target for RNA interference studies. This protein is a major mediator of apoptosis as well as inflammation and immunity, and it has...
been implicated in the pathogenesis of a wide spectrum of human diseases. Consequently, inhibition of this protein is of particular relevance. Modified oligoribonucleotides (1–3) were annealed with equimolar amounts of the unmodified guide, and the resulting duplexes were used to inhibit the expression of TNF-α gene. HeLa cells were transfected first with the murine TNF-α plasmid using lipofectin, and 1 h later they were cotransfected with the siRNA duplex using oligofectamine. After 24 h, cellular TNF-α production was analyzed by enzyme-linked immunosorbent assay (ELISA). The inhibitory capacity of the siRNA duplexes is shown in Figure 6. To compare the efficiency of each siRNA to inhibit TNF-α, we normalized the data taking into account the number of strands of each siRNA. Thus, a 100 nM double strand concentration is equivalent to 100 nM of unmodified siRNA duplex, 50 nM of siRNA 1, and 2 and 25 nM of siRNA 3. Figure 6 shows that the inhibitory capacity of the branched structures was maintained similar to that of the unmodified duplex. This result indicates that the branched siRNAs described here are compatible with RNA interference machinery, and thus the RISC complex binds to branched RNA structures in a similar way as to the linear RNA duplexes shown in Figure 1. Two-stranded RNA duplexes (1 and 2) were more efficient than four-stranded ones (3). In addition, siRNA 1 showed slightly greater efficiency at inhibiting TNF-α than siRNA duplex 2. This small difference may be related to the lower melting temperature of the former (Table 1).

### 4. Conclusions

For several years, research has focused on chemical modifications and delivery technologies to improve the pharmacokinetic properties of siRNA. Many of the chemically modified siRNA with interesting inhibitory capacity contain one or multiple modifications in the sugar, nucleobases, and phosphate linkages or at the 3′- or 5′-ends. In addition to these modifications, duplex architecture of siRNA itself is also relevant, and several modifications have been reported to show satisfactory inhibitory capacity. Here we demonstrate that branched siRNA is compatible with RNAi and that, when transfected with cationic lipids, siRNA has similar inhibitory capacity than unmodified duplex siRNA. Although the potency of branched siRNA containing two or four strands was not increased, we consider it a suitable starting point for further development. Given that the method described here is compatible with most of the RNA modifications described to date, these compounds may be further functionalized to obtain more potent siRNA derivatives. In addition, they offer an internal mid position that could be suitable for attachment to delivery systems. In this regard, optimization of the branching approach for the synthesis of asymmetric branched siRNAs may lead to the development of siRNA for the combined inhibition of multiple targets. These asymmetric siRNA duplexes carrying two RNA sequences attached or bound to an appropriate delivery system will insure the 1:1 ratio of two RNA sequences for the combined inhibition of two genes that may improve the treatment of a particular disease.

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### References


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### Table 1: Mass spectrometry data on modified passenger strand and melting temperatures of siRNA duplexes formed by oligoribonucleotides 1, 2, and 3 and the linear unmodified control sequences. Buffer conditions: 15 mM HEPES, 1 mM magnesium acetate, 50 mM potassium acetate pH 7.4.

<table>
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<th>N°</th>
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<th>MS (expected)</th>
<th>MS (found)</th>
<th>Tm (°C)</th>
<th>ΔT (°C)</th>
</tr>
</thead>
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<td>13504</td>
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</tr>
<tr>
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<td>(5′TNFa 3′)2-DB-dT</td>
<td>13842</td>
<td>13847</td>
<td>82.5</td>
<td>−1.7</td>
</tr>
<tr>
<td>3</td>
<td>(5′TNFa 3′)′2-(DB)2-DB-dT</td>
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<td>27823</td>
<td>84.3</td>
<td>+0.1</td>
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<tr>
<td></td>
<td>Unmodified Passenger TNFa</td>
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<td>n.d.</td>
<td>84.2</td>
<td>0</td>
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</table>
Review Article

tRNA/mRNA Mimicry by tmRNA and SmpB in Trans-Translation

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Since accurate translation from mRNA to protein is critical to survival, cells have developed translational quality control systems. Bacterial ribosomes stalled on truncated mRNA are rescued by a system involving tmRNA and SmpB referred to as trans-translation. Here, we review current understanding of the mechanism of trans-translation. Based on results obtained by using directed hydroxyl radical probing, we propose a new type of molecular mimicry during trans-translation. Besides such chemical approaches, biochemical and cryo-EM studies have revealed the structural and functional aspects of multiple stages of trans-translation. These intensive works provide a basis for studying the dynamics of tmRNA/SmpB in the ribosome.

1. Introduction

Translation from the genetic information contained in mRNA to the amino acid sequence of a protein is performed on the ribosome, a large ribonucleoprotein complex composed of three RNA molecules and over 50 proteins. The ribosome is a molecular machine that catalyzes the synthesis of a polypeptide from its substrate, aminoacyl-tRNA. Ribosomes that translate a problematic mRNA, such as that lacking a stop codon, can stall at its 3′ end and produce an incomplete, potentially deleterious protein. Trans-translation is known as the highly sophisticated system in bacteria to recycle ribosomes stalled on defective mRNAs and add a short tag-peptide to the C-terminus of the nascent polypeptide as the degradation signal [1–4] (Figure 1). Thus, the tagged polypeptide from truncated mRNA is preferentially degraded by cellular proteases including ClpXP, ClpAP, Lon, FtsH, and Tsp [1, 5–7], and truncated mRNA is released from the stalled ribosomes to be degraded by RNases [8]. The process of trans-translation is facilitated by transfer-messenger RNA (tmRNA, also known as 10Sa RNA or SsrA RNA), which is a unique hybrid molecule that functions as both tRNA and mRNA (Figure 2). It comprises two functional domains, the tRNA domain partially mimicking tRNA [9] and the mRNA domain, which includes the coding region for the tag-peptide, surrounded by four pseudoknot structures [10–14]. As predicted from the tRNA-like secondary structure, the 3′ end of tmRNA is aminoacylated by alanyl-tRNA synthetase (AlaRS) like that of canonical tRNA [15, 16]. The function as tRNA is a prerequisite for the function as mRNA, indicating the importance of the elaborate interplay of the two functions [2]. Thus, “trans-translation” has been proposed: Ala-tmRNA somehow enters the stalled ribosome, allowing translation to resume by switching the original mRNA to the tag-encoding region on tmRNA. Various questions about the molecular mechanism of this process have been raised. How does tmRNA enter the stalled ribosome in the absence of a codon-anticodon interaction? How is tmRNA switched from the original mRNA in the ribosome? How is the residue codon on tmRNA for the tag-peptide determined? How does tmRNA, 4- or 5-fold larger than tRNA, work in the narrow space in the ribosome?

Several factors, including EF-Tu [17–20], SmpB [21–23], and ribosomal protein S1 [22–24], have been identified as tmRNA-binding proteins. EF-Tu delivers Ala-tmRNA to the ribosome like aminoacyl-tRNA in translation. Unlike S1 [25–27], SmpB serves as an essential factor for trans-translation in vivo and in vitro. It binds to the tRNA-like domain (TLD) of tmRNA [23, 28–30] and ribosome [21] to perform multiple functions during trans-translation, including enhancement of aminoacylation efficiency of tmRNA...
[22, 23, 31], protection of tmRNA from degradation in the cell [19, 28], and recruitment of tmRNA to the stalled ribosome [21, 23]. NMR studies have revealed that SmpB consists of an antiparallel β-barrel core with three helices and flexible C-terminal tail residues that are disordered in solution [32, 33].

Here, we review recent progress in our understanding of the molecular mechanism of trans-translation facilitated by tmRNA and SmpB, which is being revealed by various chemical approaches such as directed hydroxyl radical probing and chemical modification as well as other biochemical and structural studies.

2. In Vitro Trans-Translation System

A cell-free trans-translation system coupled with poly (U)-dependent polyphenylalanine synthesis was developed using Escherichia coli crude cell extracts [2]. Later, several trans-translation systems were developed using purified factors from E. coli [31, 34, 35] or from Thermus thermophilus [25]. These systems have revealed that EF-Tu and SmpB, in addition to the stalled ribosome and Ala-tmRNA, are essential and sufficient for the first few steps of trans-translation including the binding of Ala-tmRNA to the ribosome, peptidyl transfer from peptidyl-tRNA to Ala-tmRNA, and decoding of the first codon on tmRNA for the tag peptide. Besides, these systems have also provided a basis for investigating the molecular mechanism of trans-translation by chemical approaches.

3. Molecular Mimicries of tRNA and mRNA Revealed by Directed Hydroxyl Radical Probing

Ivanova et al. [36] performed chemical probing to analyze the interaction between SmpB and a ribosome. Bases of rRNA are protected from chemical modification with dimethylsulfate or kethoxal by SmpB, indicating that there are two SmpB-binding sites on the ribosome; one is around the P-site of the small ribosomal subunit and the other is under the L7/L12 stalk of the large ribosomal subunit. The capacity of two SmpB molecules to bind to a ribosome is in agreement with results of other biochemical studies [37, 38]. Gutmann et al. [29] showed a crystal structure of Aquifex aeolicus SmpB in complex with the tmRNA fragment corresponding to TLD, which confirmed results of earlier biochemical studies showing that TLD is the crucial binding region of SmpB [23]. It also suggested that SmpB orients toward the decoding center of the small ribosomal subunit and that SmpB structurally mimics the anticodon arm. This is in agreement with a cryo-EM map of the accommodated state complex of ribosome/Ala-tmRNA/SmpB [39–41].

A truncation of the unstructured C-terminal tail of SmpB leads to a loss of trans-translation activity [42, 43]. In spite of its functional significance, cryo-EM studies have failed to identify the location of the C-terminal tail of SmpB in the ribosome due to poor resolution. We performed directed hydroxyl radical probing with Fe(II)-BABE to study the sites and modes of binding of E. coli SmpB to the ribosome (Figure 3). Fe(II)-BABE is a specific modifier of the cysteine residue of a protein, which generates hydroxyl radicals to cleave the RNA chain. Cleavage sites on RNA can be detected by primer extension, allowing mapping of amino acid residues of a binding protein on an RNA-based macromolecule. This is an excellent chemical approach to study the interaction of a protein with the ribosome [44–47]. We prepared SmpB variants each having a single cysteine residue for attaching it to an Fe(II)-BABE probe. Using directed hydroxyl radical probing, we succeeded in identifying the location of not only the structural domain but also the C-terminal tail of SmpB on the ribosome [48].

It was revealed that there are two SmpB-binding sites in a ribosome, which correspond to the lower halves of the A-site and P-site and that the C-terminal tail of A-site SmpB is aligned along the mRNA path towards the downstream tunnel, while that of P-site SmpB is located almost exclusively around the region of the codon-anticodon interaction in the P-site. This suggests that the C-terminal tail of SmpB mimics mRNA in the A-site and P-site and that these binding sites reflect the pre- and posttranslocation steps of trans-translation. The probing signals appear at interval 3 residues of the latter half of the C-terminal tail, suggesting an α-helix structure, which has been predicted from the periodical occurrence of positively charged residues [42]. Consequently, the following model has been proposed. The main body of SmpB mimics the lower half of tRNA, and the C-terminal tail of SmpB mimics mRNA both before and after translocation, while the upper half of tRNA is mimicked by TLD. Upon entrance of tmRNA into the stalled ribosome, the C-terminal tail of SmpB may recognize the vacant A-site free of mRNA to trigger trans translation. After peptidyl transfer to Ala-tmRNA occurring essentially in the same manner as that in canonical translation, translocation of peptidyl-Ala-tmRNA/SmpB from the A-site to the P-site may occur. During this event, the extended C-terminal tail folds around the region of the codon-anticodon interaction in the P-site, which drives out mRNA from the P-site.

4. Early Stages of Trans-Translation

Ala-tmRNA/SmpB forms a complex with EF-Tu and GTP in vitro, and this quaternary complex is likely to enter the empty A-site of the stalled ribosome [22]. This complex forms an initial binding complex with the stalled ribosome like the ternary complex of aminoacyl-tRNA, EF-Tu, and GTP does with the translating ribosome. In normal translation, the correct codon-anticodon interaction is recognized by universally conserved 16S rRNA bases, G530, A1492 and A1493, which form the decoding center. When a cognate tRNA binds to the A-site, A1492, and A1493 flip out from the interior of helix 44 of 16S rRNA, and G530 rotates from a syn to an anticonformation to monitor the geometry of the correct codon-anticodon duplex [53]. This induces GTP hydrolysis by EF-Tu, allowing the CCA terminal of tRNA to be accommodated into the peptidyl transferase center. In the context of tRNA mimicry, SmpB should orient toward the decoding center in trans-translation. We have recently
shown that interaction of the C-terminal tail of SmpB with the mRNA path in the ribosome occurs after hydrolysis of GTP by EF-Tu [49]. According to a chemical probing and NMR study, SmpB interacts with G530, A1492, and A1493 [54]. How these bases recognize SmpB to trigger the following GTP hydrolysis is yet to be studied. It should be noted that recent crystal structures have revealed that these bases recognize the A-site ligands (aminoacyl-tRNAs, IF-1, RF-1, RF-2 and RelE) in different ways during translation [50, 55, 56].

Cryo-EM reconstructions of the preaccommodated state of the ribosome/Ala-tmRNA/SmpB/EF-Tu/GDP/kirromycin complex of *T. thermophilus* have shown that two SmpB molecules present in a complex, one binding to the 50S ribosomal subunit at the GT-Pase-associated center and the other binding to the 30S subunit near the decoding center [39, 41]. The latter SmpB is not found in the accommodation complex of *T. thermophilus* and *E. coli* [39–41]. Thus, the following model has been proposed: two molecules of SmpB are required for binding of Ala-tmRNA to the stalled ribosome and one of them is released from the ribosome concomitant with the release of EF-Tu after hydrolysis of GTP, so that the 3′-terminal of tmRNA is oriented toward the peptidyl-transferase center. However, several reports have argued against the requirement of two SmpB molecules for *trans*-translation: SmpB has been reported to interact with tmRNA in a 1 : 1 stoichiometry in the cell [57, 58], and crystal structures of SmpB in complex with TLD have been reported to exhibit a 1 : 1 stoichiometry of tmRNA and SmpB [29, 59]. Further studies are required to assess the stoichiometry of SmpB in the preaccommodation state complex.

We have recently shown that the C-terminal tail of SmpB is required for the accommodation of Ala-tmRNA/SmpB into the A-site rather than the initial binding of Ala-tmRNA/SmpB/EF-Tu/GTP to the stalled ribosome [49]. We have also shown that the tryptophan residue at 147 in the middle of the C-terminal tail of *E. coli* SmpB has a crucial role in the step of accommodation. Our results further suggest that the aromatic side chain of Trp147 is required for interaction with rRNA upon accommodation.

It has been shown that *trans*-translation can occur in the middle of an mRNA *in vitro*, although the efficiency of *trans*-translation is dramatically reduced with increase in the length of the 3′ extension from the decoding center [34, 35]. This may be a result of competition of the 3′ extension of mRNA and the C-terminal of A-site SmpB for the mRNA path. The ribosome stalled on the middle of intact mRNA in a cell might be rescued by *trans*-translation via cleavage of
Figure 2: Secondary structure model of tmRNA from E. coli. The tRNA-like domain and mRNA domain are highlighted with red and blue, respectively. The tag-encoding sequence is surrounded by four pseudoknot structures (PK1-4).
mRNA at the A-site [60] or by alternative ribosome rescue systems [61–63].

5. Determination of the Resume Codon

In trans translation, the ribosome switches template from a problematic mRNA to tmRNA. How does the stalled ribosome select the first codon on tmRNA without an SD-like sequence? It is reasonable to assume that some structural element on tmRNA is responsible for positioning the resume codon in the decoding center just after translocation of peptidyl-Ala-tmRNA/SmpB from the A-site to the P-site. In E. coli, the coding region for the tag peptide starts from position 90 of tmRNA, which is 12 nucleotides downstream of PK1. Indeed, PK1 is important for efficiency of trans-translation [14], whereas changing the span between PK1 and the resume codon does not affect determination of the initiation point of tag-translation [64]. A genetic selection experiment has revealed strong base preference in the single-stranded region between PK1 and the resume codon, especially −4 and +1 (position 90) [65]. The importance of this region has also been shown by an in vitro study [64]. Several point mutations in this region encompassing −6 to −1 decrease the efficiency of tag-translation, while some of them shift the tag-initiation point by −1 or +1 to a considerable extent [59, 60], indicating that the upstream sequence contains not only the enhancer of trans-translation but also the determinant for the tag-initiation point. Evidence for interaction between the upstream region and SmpB has been provided by a study using chemical probing [66]. E. coli SmpB protects U at position −5 from chemical modification by CMCT. The structural domain of SmpB rather than the C-terminal tail is involved in this protection. The protection at −5 was suppressed by a point mutation in the TLD critical to SmpB binding, suggesting that SmpB serves to bridge two separate domains of tmRNA to determine the resume codon for tag-translation. Mutations that cause −1 and +1 shifts of the start point of tag-translation also shift the site of protection at −5 from chemical modification by −1 and +1, respectively, indicating the significance of the fixed span between the site of interaction on tmRNA with SmpB and the resume point of translation: translation for the tag-peptide starts from the position 5 nucleotides downstream of the site of interaction with SmpB. Such a functional interaction of the upstream region in tmRNA with SmpB is also supported by the results of another genetic study showing that A-to-C mutation at position 86 of E. coli tmRNA that inactivates trans-translation both in vitro and in vivo is suppressed by some double or triple mutations in SmpB [67]. In agreement with these studies, recent cryo-EM studies have suggested that the upstream region in tmRNA interacts with SmpB in the resume (posttranslocation) state [68, 69].

The initiation shift of tag-translation can also be induced by the addition of a 4,5- or 4,6-disubstituted class of aminoglycoside such as paromomycin or neomycin [70, 71], which usually causes miscoding of translation by binding to the decoding center on helix 44 of the small subunit to induce a conformational change in its surroundings [72]. Aminoglycosides also bind at helix 69 of the large subunit, which forms the B2a bridge with helix 44 in close proximity of the decoding center in the small subunit, to inhibit translocation and ribosome recycling by restricting the helical dynamics of helix 69 [73]. Taken together, these findings suggest the significance of interaction of the proximity of the decoding center with any portion of SmpB or tmRNA for precise tag-translation. It should be noted that hygromycin B, which binds only to helix 44, does not induce initiation shift of tag-translation [71].

6. Trajectories of tmRNA/SmpB

Along with the functional mimicry of TLD/SmpB, a similar behavior of tmRNA/SmpB to that of canonical tRNA+mRNA in the ribosome through several hybrid states,
A/T, A/A, A/P, P/P, and P/E, has been assumed. Cryo-EM studies have shown the location of the complex of tmRNA with the main body of SmpB in the A/T and A/A states [39, 40], and a directed hydroxy radical probing has revealed the positions of SmpB in the A/A and P/P states [48]. The existence of stable SmpB binding sites in the A-site and P-site suggests the requirement of translocation, as in canonical translation. It might possibly involve EF-G. Concomitantly with translocation, mRNA and P-site tRNA are released from the stalled ribosome [74]. Considering the different C-terminal tail structures of A-site SmpB and P-site SmpB, the C-terminal tail would somehow undergo conformational change from the extended form to the folded form [48].

The next translocation is thought to move tmRNA/SmpB to the E-site. These ribosomal processes should involve extensive changes in the conformation of tmRNA [75] as well as in the modes of interactions of tmRNA with SmpB and the ribosome [76, 77]. According to chemical probing studies, secondary structure elements of tmRNA remain intact in a few steps of trans-translation including pre- and posttranslocation states [77–79]. Another study has suggested 1:1 stoichiometry of tmRNA to SmpB throughout the processes of translation for the tag peptide [80]. Recently, the movement of tRNA during translocation has been revealed by using time-resolved cryo-EM [81]. Not only classic and hybrid states but also various novel intermediate states of tRNAs were revealed. Although the intermediate states during trans-translation remain unclear, results of future structural studies including chemical approaches should reveal tmRNA/SmpB and ribosome dynamics.

Figure 4: A model of the early stages of trans-translation. The C-terminal tail of SmpB is not located on the mRNA path in the processes before accommodation. After GTP hydrolysis by EF-Tu, the C-terminal tail is located on the mRNA path mimicking mRNA to recognize the stalled ribosome free of mRNA. Following translocation of tmRNA/SmpB from the A-site to P-site, the C-terminal tail undergoes drastic conformational change to accommodate the resume codon of tmRNA into the decoding center. SmpB and the tag-encoding region are shown by red and blue, respectively. White circles indicate amino acids encoded by truncated mRNA, and a white square indicates amino acid designated by the resume codon of tmRNA.

### 7. Conclusion

Various chemical approaches in addition to cryo-EM and X-ray crystallographic studies have been revealing the molecular mechanism of trans-translation. tmRNA forms a ribonucleoprotein complex with SmpB, which plays an essential role in trans-translation. Based on a directed hydroxyl radical probing towards SmpB, we have proposed a novel molecular mechanism of trans-translation (Figure 4). In this model, an elegant collaboration of a hybrid RNA molecule of tRNA and mRNA and a protein mimicking a set of tRNA and mRNA facilitates trans-translation. Initially, a quaternary complex of Ala-tmRNA, SmpB, EF-Tu, and GTP may enter the vacant A-site of the stalled ribosome to trigger trans-translation, when a set of Ala-TLD of tmRNA and the main body of SmpB mimicking the upper and lower halves of aminoacyl-tRNA, respectively, recognizes the A-site free of tRNA. After hydrolysis of GTP by EF-Tu, the C-terminal tail of SmpB mimicking mRNA interacts with the decoding center and the downstream mRNA path free of mRNA, allowing Ala-TLD/SmpB to be accommodated. While several proteins including SmpB have been proposed to mimic tRNA or its portion, SmpB is the first protein that has been shown to mimic mRNA. SmpB is also the first protein of which stepwise movements in the ribosome are assumed to mimic those of tRNA in the translating ribosome.
Our model depicts an outline of the trans-translation processes in the ribosome, although the following issues should be addressed. How do the intermolecular interactions between tmRNA and ribosome, between tmRNA and SmpB, and between ribosome and SmpB as well as the intramolecular interactions within tmRNA and within SmpB change during the course of the trans-translation processes? Is EF-G required for translocation of tmRNA/SmpB having neither an anticodon nor the corresponding codon from the A-site to the P-site? If EF-G is required, how does it promote translocation? These questions remain to be answered in the future works.

Abbreviations

AlaRS: Alanyl-tRNA synthetase
BABE: 1-(p-bromoacetamidobenzyl)-ethylenediaminetetraacetic acid
CMCT: 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate
cryo-EM: Cryo-electron microscopy
EF-Tu: Elongation factor Tu
EF-G: Elongation factor G
PK: Pseudoknot
TLD: tRNA-like domain.

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