

# Sequence Analysis of the Mouse RAG Locus Intergenic Region

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The recombination activating genes RAG-1 and RAG-2 are highly conserved throughout evolution and are necessary and essential for the DNA rearrangement of antigen-receptor gene segments. These convergently transcribed genes are expressed primarily by developing B and T lineage cells. In addition, recent data suggest that the RAG locus can be reactivated in mouse germinal center B cells. Despite these well-defined patterns of expression, little is known about mechanism(s) regulating transcription of the RAG locus. Experiments with a mouse fibroblast line stably transfected with a genomic fragment of the RAG locus suggest that the intergenic region between RAG-1 and RAG-2 may contain information modulating RAG transcription. In order to begin testing this hypothesis, we have sequenced the 7.0-kb RAG intergenic region of the mouse. The sequence did not contain open reading frames larger than 60 amino acids. Analysis with GCG software identified several potential transcription-factor binding sequences within this region. Many of these are associated with transcriptional regulation of the Ig locus.

**Keywords:** RAG-1, RAG-2, lymphopoiesis, regulation, promoter, enhancer

## INTRODUCTION

Genes encoding antigen receptors are assembled through somatic DNA rearrangement of the gene segments encoding the variable portions of the immunoglobulin molecule (see Lewis, 1994, for review). Typically, this process occurs during discrete stages of lymphocyte development in either the bone marrow for B cells or the thymus for T cells. The recombination activating genes, RAG-1 and RAG-2,

are necessary and essential for this process (Oettgen et al., 1990; Mombaerts et al., 1992; and Shinkai et al., 1992).

The coordinately transcribed RAG-1 and RAG-2 genes are usually expressed together. Only in the chicken bursa (RAG-2 only) and in the mouse brain (RAG-1) are one of the RAG genes expressed without the other (Chun et al., 1991; Takeda et al., 1992). The expression of the RAG genes varies throughout lymphopoiesis. In murine B lineage development,

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high levels of RAG are found in the earliest stages of B lineage development when heavy-chain rearrangement initiates (B220<sup>+</sup> CD43<sup>+</sup> cells) and then decreases through the cytoplasmic  $\mu^+$  stage. RAG levels rise again during the onset of light-chain rearrangement (Li *et al.*, 1993). A similar pattern is seen in human B-lineage development (Ghia *et al.*, 1996). In more mature B cells, the RAG locus is inactive. However, RAG expression has recently been demonstrated in germinal-center B cells of mice that have undergone immunization (Han *et al.*, 1996; Hikida *et al.*, 1996), suggesting a role for the RAG proteins in later aspects of B-lineage development, such as receptor editing.

Several studies have indicated that RAG transcription can be modulated through pathways involving protein kinase A (PKA), protein kinase C (PKC), and cAMP. Increases in cAMP result in an increase of RAG transcription, acting through the PKA pathway. Induction of the PKC pathway will decrease RAG transcription (Menetski and Gellert, 1990; Casillas *et al.*, 1995). However, the *cis*-acting elements involved in this modulation of RAG locus are unknown.

Using the fibroblast line L4, which contains a genomic fragment of the RAG locus under the control of the SV2 promoter (Schatz *et al.*, 1989), Dobbeling and colleagues showed that transcription from this fragment can be influenced via the PKA and PKC pathway in the same way that these second messengers influenced transcription from the endogenous RAG locus in a pre-B cell line (Dobbeling *et al.*, 1996). Moreover, the genomic fragment in the L4 fibroblast line was lacking the region 5' of RAG-2 (Schatz *et al.*, 1989). This result suggests that elements modulating RAG locus transcription through these second messenger pathways lie 5' of RAG-1 and/or in the intergenic region between the two genes.

On the basis of these data, and because the RAG genes are relatively close together (7.0 kb in the mouse, 2.6 kb in the zebrafish), we hypothesized that the intergenic region in between RAG-1 and RAG-2 may contain sequence element(s) involved in coordinately modulating transcription of the RAG locus. In order to investigate this possibility, we have sequenced the murine 7.0-kb RAG intergenic region.

Analysis with GCG software has revealed a variety of potential regulatory elements in this region.

## MATERIALS AND METHODS

### Cloning of the Murine RAG Intergenic Region

The clone pJH493, which encompasses most of RAG-1, the intergenic region, and RAG-2, was the kind gift of Dr. J. Hesse, NIH. Two overlapping fragments of the intergenic region were subcloned from the pJH493 plasmid. Fragment 1 (4.0 kb) was generated by digesting pJH493 with Bgl II and cloning the fragment into the Bam H I site of pBluescript (Stratagene, La Jolla, CA). Fragment 2 (3.5 kb) was generated by digesting the pJH493 with EcoR V and subcloning into the Sma I site of pBluescript (Stratagene, La Jolla, CA).

### Sequence Analysis

Single-stranded sequence of the Fragment 1 and Fragment 2 clones was obtained using a Pharmacia A.L.F. automatic sequencer (HSC Biotech. Centre, U. Toronto). Sequences were assembled using DNASTrider and analyzed for transcription binding-factor motifs using the TFSITES database and GCG software. Homology searches were done using the NIH Blast algorithm for searching GenBank. This sequence has been deposited in GenBank under the accession number U96151. GRAIL software developed by ORNL (Oak Ridge National Laboratory, Oak Ridge, TN) was used to assess the region for potential open reading frames.

## RESULTS

### Sequence of the Murine RAG Intergenic Region

The murine RAG intergenic region ( $\sim$  7 kb) was subcloned from the pJH493 clone of the RAG locus, as two overlapping fragments 4.0 and 3.5 kb, respectively (Figure 1). Single-stranded sequence of each fragment was obtained as described in Materials and Methods. This sequencing revealed the RAG

intergenic region (defined here as beginning immediately 3' of the published 3' UTR sequence of RAG-1 and RAG-2) to be 7004 base pairs in length. The intergenic region is illustrated in Figure 1 and has been deposited into GenBank under the accession number U96151. Figure 2 lists the sequence. Three-phase amino acid translation analysis of this region failed to reveal any open reading frames. The GRAIL computer algorithm, designed to detect open reading frames (ORFs) in genomic sequences was also used to examine this region for the ability to encode a gene. In agreement with the translation, no potential ORFs

were identified. This result is consistent with previous Northern analysis of this region (Schatz et al., 1989).

### Potential Transcription-Factor Binding Sites of the Murine RAG Intergenic Region

GCG software and the TFSITES database were used to examine this region for potential transcription-factor binding sites. By using a mismatch of zero, the consensus sequence for a variety of transcription-factor binding sites were identified. Many of these are

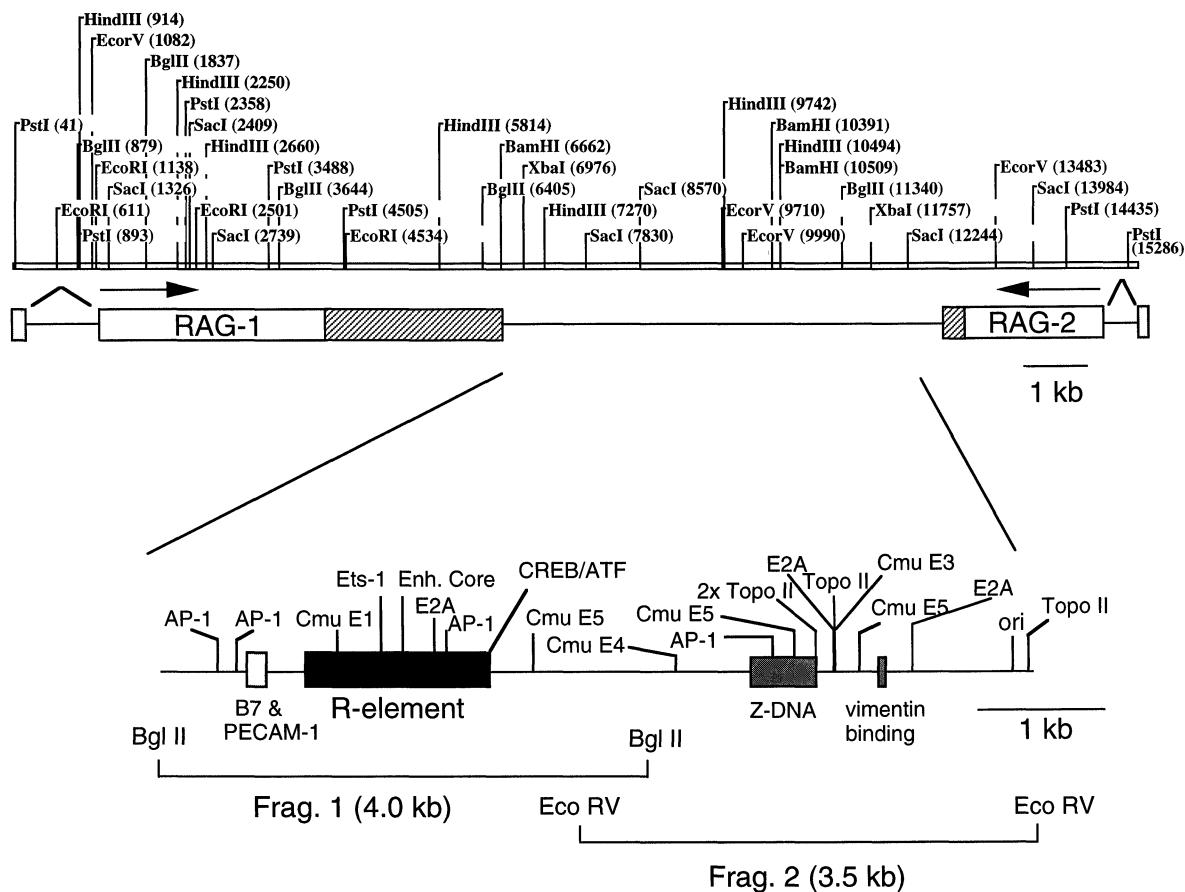


FIGURE 1 The murine RAG locus, illustrating the intergenic region. For RAG-1 and RAG-2, the open box reflects coding regions and the hatched boxes are the 3' UTRs. The expanded section is the sequenced intergenic region (accession number U96151). Potential transcription binding sites are shown above the line, and shaded boxes indicate repetitive genomic features. The top portion is a restriction map of the mouse RAG locus compiled from accession numbers M29475 (RAG-1), M54796 (RAG-2), and U96151 (the intergenic region). The numbers begin with M29475 and are numbered consecutively through RAG-2. Not included in the restriction map are the two introns immediately following 3' of the 5' UTRs of RAG-1 and RAG-2, indicated by inverted V's. Also shown are the locations of subclones Fragment 1 and Fragment 2.

known to be involved in the regulation of genes expressed in lymphopoiesis. Of note, are the AP-1 sites and the single CREB/ATF site (Figure 1), because these transcription factors have been shown to be involved in pathways regulating RAG transcription (Dobbeling et al., 1996). The locations of these sites are plotted in Figure 1 and listed in Table I. Binding sites for Cmu E5 and topoisomerase II are included in the list because of their known role in regulating the IgH enhancer, although the sequences for these deviate from their respective consensus by one or two base pairs (reviewed in Ernst and Smale, 1995).

## **Repetitive Elements Found within the RAG Intergenic Region**

A search of GenBank using the NIH BLAST algorithm revealed that the murine RAG intergenic region contains regions of homology with previously reported repetitive elements in the mouse genome. The location of these elements is shown in Figure 1. Base pairs 624-824 have sequence identity with B1-type repetitive elements found in the B7 and PECAM-1 promoters (Selvakumar et al., 1992; Almendro et al., 1996). From bases 1124-2524 is a region that is similar to murine R-elements. There are nearly 100,000 copies of R-elements in the murine genome,

FIGURE 2 Sequence of the murine RAG locus intergenic region (accession number U96151). Boldface sequences are the potential transcription-factor binding sites listed in Table 1.

TABLE I Transcription-Factor Binding Motifs in the Murine RAG Intergenic Region<sup>†</sup>

| Transcription Factor | 5' end | Sequence               |
|----------------------|--------|------------------------|
| AP-1                 | 427    | TGAGTCAC               |
| AP-1                 | 619    | TGATTAA                |
| Cu E1.2              | 1395   | GATGCCG                |
| Ets-1                | 1731   | ACTTCCGG               |
| Enhancer core        | 1815   | GTGGAATG               |
| E2A                  | 2137   | GCAGCTG                |
| AP-1                 | 2173   | TTAGTCAC               |
| CREB/ATF             | 2615   | TGACGT                 |
| Cmu E5               | 2806   | TGCA <del>t</del> GTGT |
| Cmu E4               | 3943   | ACCACCTG               |
| AP-1                 | 4745   | TTAGTCAG               |
| Cmu E5               | 4866   | TaCAGGTGT              |
| Topoisomerase II/Rev | 4886   | CAAATGgAcATACAC        |
| Topoisomerase II/Rev | 4908   | CACgTGtATGTACAC        |
| E2A                  | 5311   | ACAGATG                |
| Topoisomerase II     | 5464   | GTGTgTATcCATATG        |
| Cmu E3               | 5489   | GTCATGTGGC             |
| Cmu E5               | 5503   | TGCA <del>t</del> GTGT |
| E2A                  | 5572   | CAGCTGC                |
| Topoisomerase II     | 6888   | CTCATAAgTATAAAC        |

<sup>†</sup>Indicated are selected transcription-factor binding sites and the location of the 5' end of each sequence. These sites correspond to the sequences in boldface in Figure 1. Numbering refers to GenBank accession number U96151. Mismatches from the consensus are indicated by lowercase letters.

found in association with a variety of genes, including the  $\beta$ -globin locus and the Ig light-chain loci (Gebhard et al., 1972; Gebhard and Zachau, 1983). It is interesting to note that many of these repetitive elements are associated with the promoters of a variety of genes, and that the potential transcription-factor binding sites seem to cluster around the location of the repetitive elements.

### Regions of Possible Matrix Attachment within the RAG Intergenic Region

The intergenic region from bp 4500 to 6800 contains sequences associated with matrix attachment and unusual DNA structures. This region contains a sequence with ~60% homology with the consensus sequence for the binding site of the intermediate filament vimentin (Figure 1). This protein is thought to selectively anchor areas of GC-rich DNA to areas of matrix attachment (Wang et al., 1996). 5' and 3' of the potential vimentin binding sequence are topoisomerase II sites, which are associated with regions of matrix attachment (Gasser et al., 1989, and references

therein). Moreover, three T-box motifs (TTWTWTTWTT) are found between bp 5000 and 5590, which are also associated with matrix attachment (Gasser et al., 1989, and references therein). Nearby, there is a region with homology to Z-DNA motifs found in the nitric oxide synthase gene promoter, TCR V $\gamma$ 1 and V $\gamma$ 2 promoter, and the  $\mu$ - $\delta$  intron (Milstein et al., 1984; Kuziel et al., 1994; Eberhardt et al., 1996).

### Sequence Homologies with the RAG Intergenic Region of Other Species

Recently, the RAG intergenic region of the zebrafish and the trout have been sequenced (Bertrand et al., in press; Hansen, in press). A comparison of these sequences reveals that there is limited sequence homology between the teleost and the murine RAG intergenic regions (data not shown). This is perhaps not surprising because the intergenic region of the fish is also likely the 3' UTR of RAG-1 and RAG-2 (Bertrand et al., 1997; Hansen, in press; Willett et al., 1997). However, no homologies were found between

the murine RAG-1 and RAG-2 3' UTR and the two teleost RAG intergenic regions. Moreover, the murine RAG-1 and RAG-2 3' UTRs do not contain the wide array of Ig-related transcription-factor motifs observed in the intergenic region (data not shown).

Although there is limited homology among the mouse RAG 3' UTRs, the mouse intergenic region, and the analogous region in the teleost, an area of the trout and zebrafish intergenic region share 50% similarity (Figure 3). This region contains motifs (mismatch = 1) for the transcription factors Cmu E4, CREB, Cmu E5, AP-1, E2A, Ets-1, and an enhancer core element, which are also found in the murine intergenic sequence. Thus, although there is no strong sequence homology between this region and murine

RAG intergenic region, many of the potential regulatory sites are in common.

## DISCUSSION

The 7.0-kb murine RAG locus intergenic region has been sequenced and analyzed for potential *cis*-regulatory motifs. Several motifs known to be involved in the regulation of the IgH enhancer (reviewed in Ernst and Smale, 1995) and motifs involved in second messenger pathways that modulate RAG transcription (Menetski and Gellert, 1990; Casillas et al., 1995; Dobbeling et al., 1996) are contained within this sequence. However, preliminary

|        | v670   | v680 | v690 | v700 | v710 | v720 |
|--------|--|------|------|------|------|------|
| Trout  | TTTATTGTATTGCATGGGTTATGACACATCCATGAATTTCAGT-GGGTTTATTTATG    |      |      |      |      |      |
| Z-fish | TTT TTGTATTGC GGTT TGACAC T CA GAATT AT CAG GGGTT ATTTATG    |      |      |      |      |      |
|        | TTTTTTGTATTGCCA-GGTTGTGACACCTTCACGAATTATGCAGAAGGGTT-ATTTATG  |      |      |      |      |      |
|        | ▲270 ▲280 ▲290 ▲300 ▲310 ▲320                                |      |      |      |      |      |
|        | v730 v740 v750 v760 v770 v780                                |      |      |      |      |      |
| Trout  | GGAACAAATGAAAGCACTGTAAGTCATGGAATGGAATTGTGATAAAAGGAGC-TCACATT |      |      |      |      |      |
| Z-fish | AAC A GAA G TGTAAGTC GAA ATT T TAAAGGAGC TCAC TTT            |      |      |      |      |      |
|        | AAAACGTACGAAGGAGTTGTAAGTCACTGAA---ATTATATTAAAGGAGCCTCACGTTT  |      |      |      |      |      |
|        | ▲330 ▲340 ▲350 ▲360 ▲370 ▲380                                |      |      |      |      |      |
|        | v790 v800 v810 v820 v830                                     |      |      |      |      |      |
| Trout  | ATGATCAGTGATTAG--TTTATCTCCAGTCCTGGAGCAAAATGGAATCATATAGACTC-  |      |      |      |      |      |
| Z-fish | ATGATTGCTATATTAATATTTCACA-CAGGGACAGAGACAAAGTGAAT---TAGAAAAT  |      |      |      |      |      |
|        | ▲390 ▲400 ▲410 ▲420 ▲430                                     |      |      |      |      |      |
|        | v850 v860 v870 v880  |      |      |      |      |      |
| Trout  | ---AAATTGTCTTC--GTTTCAGGCTGCCATCTAAAAA-----TATTGCTTTAGAGCT   |      |      |      |      |      |
| Z-fish | AAATT C GTTT AGG AAA TATT TTTA T                             |      |      |      |      |      |
|        | GTAAATTGCAGGAGGTTGAGGG-----AACAGGGGTTATT--TTTACCAAT          |      |      |      |      |      |
|        | ▲440 ▲450 ▲460 ▲470 ▲480                                     |      |      |      |      |      |
|        | v890 v900 v910 v920 v930                                     |      |      |      |      |      |
| Trout  | TCAT-ATTGGGTCTGAAGTCCAAAATGGGGCCTG---GGT-----GACTTG---       |      |      |      |      |      |
| Z-fish | CAT ATTT TGA TGG GGC GGT GACT                                |      |      |      |      |      |
|        | GCATTATTT-----TGA-----TGGTGGCTCAAATKGGTCACAATGACTGAATGC      |      |      |      |      |      |
|        | ▲490 ▲500 ▲510 ▲520 ▲530                                     |      |      |      |      |      |
|        | v940 v950 v960   |      |      |      |      |      |
| Trout  | AAACCATCTTATCTGTTTAGCATTGCA                                  |      |      |      |      |      |
| Z-fish | AAA C TAT AGCATTGCA  |      |      |      |      |      |
|        | AAATAGACACTATGGTAC--AGCATTGCA                                |      |      |      |      |      |
|        | ▲540 ▲550 ▲560   |      |      |      |      |      |

FIGURE 3 The trout and zebrafish RAG intergenic regions contain a region of homology. Shown is a 290 base pair region of the zebrafish and trout RAG locus intergenic regions that is 50% similar. The middle line lists the base pairs in common. Numbering refers to accession numbers U73750 (trout) and U69610 (zebrafish).

reporter gene experiments with fragments of the intergenic region do not exhibit any enhancer activity in transient transfection assays (F. Bertrand and G. Wu, unpublished observations). Most enhancers and many promoters studied to date are associated with DNase I hypersensitive sites (Jenuwein et al., 1993, and references therein). Consistent with the lack of enhancing function in the RAG intergenic fragments, DNase hypersensitivity mapping of this locus has not identified any hypersensitive sites in the intergenic region (U. Storb, personal communication). However, this does not preclude this region from playing a modulatory role in the transcription of the RAG locus, in such a way that cannot be readily detected in transient reporter gene assays or revealed by DNase I hypersensitivity mapping.

Matrix attachment regions found within the immunoglobulin loci have been shown to play a role in the activation of the enhancers and transcriptional regulation of Ig gene segments (Cockerill and Garrard, 1986; Webb et al., 1991; Jenuwein et al., 1993). The region of the murine intergenic region from base pairs 4524 to 6908 may contain sites of matrix attachment, based on the presence of topoisomerase II sites and homology with the intermediate filament vimentin binding site. These proteins have been shown to be involved in the anchoring of DNA to regions of matrix attachment. Consistent with this idea is the presence of multiple copies of the motif (TTWTWTTWTT) in this region of the intergenic sequence. This "T-box" motif is also associated with matrix attachment regions (Gasser et al., 1989). The close proximity of a Z-DNA sequence motif, which may provide for an unusual DNA structure in this region, is also compelling. Thus, this region may contain regulatory features of the murine RAG locus that are based on DNA structure and accessibility. This type of regulation awaits a more detailed biochemical analysis.

Studies using fibroblasts transfected with genomic clones of the RAG locus and pre-B cell lines have demonstrated that overexpression of the transcription factor CREB results in a sharp increase of RAG transcription and the rearrangement of an exogenous plasmid substrate (Dobbeling et al., 1996). The CREB

transcription factor is involved in cAMP responses (Gonzales and Montminy, 1989). In contrast, overexpression of both c-FOS and c-JUN in these cell lines decreased RAG transcription and rearrangement of an exogenous substrate. Because overexpression of c-FOS or c-JUN alone had no effect, the repression of RAG transcription mediated by these two transcription factors likely involves AP-1 sites, which bind the FOS/JUN heterodimer (Dobbeling et al., 1996). For these reasons, we were intrigued to find AP-1 sites and a single CREB site in the RAG-locus intergenic sequence. It is possible that these sites participate in the cAMP and PKC second messenger pathways that have been shown to modulate RAG-1 and RAG-2 transcription.

Sequence comparisons of the murine RAG 3' UTRs and the intergenic region with the RAG intergenic regions of the zebrafish and trout indicate that these sequences in between the RAG genes have not been conserved during evolution, although the RAG coding regions have been. However, this circumstance does not mean that function is not preserved between these species. For instance, there is little sequence homology between the trout and mouse IgH enhancers, yet the respective regions of DNA in the two species have the same function (Magor et al., 1994). In both the zebrafish (Bertrand et al., in press) and in the mouse, there are potential binding sites for transcription factors that act in response to second messenger pathways, such as cAMP. An ~200-bp region of homology between the trout and zebrafish intergenic regions is compelling in that perhaps this sequence was preserved due to some common function. This region also contains many of the same transcription-factor motifs found in the murine intergenic sequence, suggesting a common regulatory function in the mouse and teleost.

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The sequence reported here has been deposited in GenBank under the accession number U96151.

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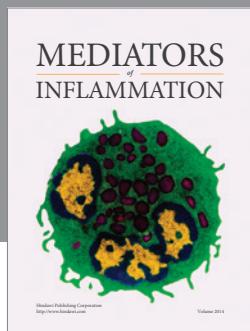
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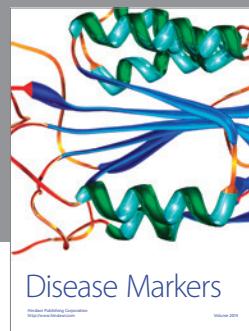
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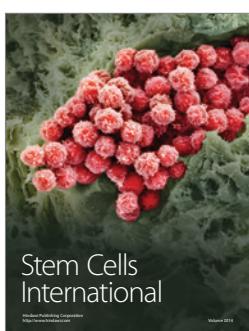
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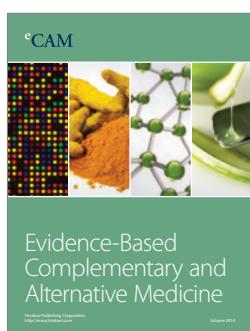
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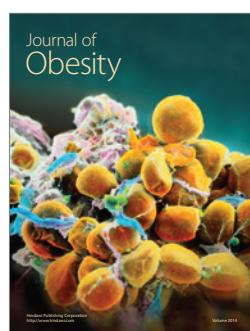
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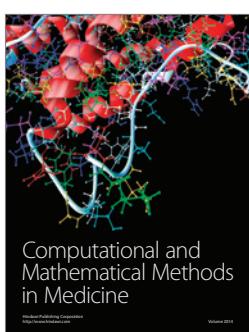
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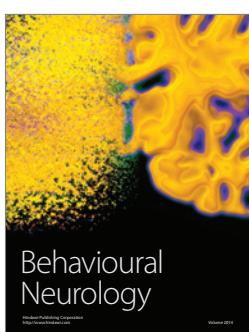
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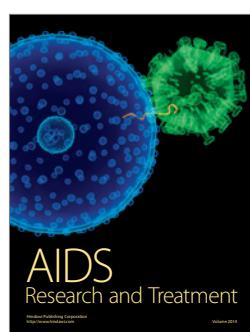
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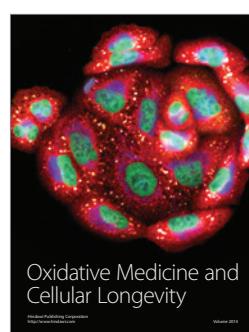
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