

# Restriction enzyme analysis of DNA methylation in “condensed” chromatin of Ha-*ras*-transformed NIH 3T3 cells

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Received February 2000

Accepted 1 October 2000

Increased amounts of chromatin condensation (i.e., localized areas of high DNA density, or chromatin higher order packing state) have been described in NIH 3T3 cells transformed with the Ha-*ras* oncogene. The structural basis for this oncogene-mediated alteration in nuclear organization is unknown. Since DNA methylation is likely to be involved in regulating the nucleosomal level of DNA packaging, we studied the role of DNA methylation in higher-order chromatin organization induced by Ha-*ras*. CpG-methylated DNA content was estimated in “condensed” chromatin of Ha-*ras*-transformed NIH 3T3 cell lines which differ in *ras* expression and *ras*-induced metastatic ability but present approximately the same values of “condensed” chromatin areas. The question posed was that if DNA methylation were involved with the chromatin higher-order organization induced by Ha-*ras* in these cell lines, the methylated DNA density in the “condensed” chromatin would also be the same. The DNA evaluation was performed by video image analysis in Feulgen-stained cells previously subjected to treatment with *Msp* I and *Hpa* II restriction enzymes, which distinguish between methylated and non-methylated DNA. The amount of methylated CpG sequences not digested by *Hpa* II

in “condensed” chromatin regions was found to vary in the studied *ras*-transformed cell lines. DNA CpG methylation status is thus suggested not to be involved with the higher order chromatin condensation induced by *ras* transformation in the mentioned NIH 3T3 cell lines.

Keywords: Chromatin higher-order condensation, Ha-*ras*, DNA methylation, restriction endonucleases, image analysis

## 1. Introduction

NIH 3T3 cells transformed by the Ha-*ras* oncogene exhibit increased levels of chromatin “condensation” in interphase nuclei as assessed by Feulgen staining and image analysis [19,20,22]. These changes are independent of the level of oncogenic *ras* expression and of *ras*-induced metastatic ability [19], but are at least in part affected by the expression of a *ras* reversion gene (lysyl oxidase, *Lox*) [22]. Furthermore, the *ras*-associated changes involving chromatin higher order packing states in rodent fibroblast cell lines were recently demonstrated to be independent of their mitotic signaling pathway [9].

Although enhancement in interphase chromatin higher order packing state is well evident in *ras*-transformed NIH 3T3 cell preparations observed by the light microscope and with video image analyzers [19–22], biochemical data have suggested that a more decondensed nucleosomal state occurs in the chromatin of these *ras*-transformed cells [13].

In other cell systems such as benzo[a]pyrene-transformed human breast epithelial cells, progressive changes in chromatin “condensation” have been demonstrated by image analysis [29]. These changes accompany genomic alterations characteristic of the *in vitro* multistep tumorigenic process [29] and were associated with nucleolar changes suggesting enhanced metabolic activities [3]. In the particular case of these transformed cells the condensed chromatin areas observed under the microscope were thus interpreted

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to represent an assembly of chromatin regions which could be defined as presenting decondensation at the nucleosomal level [29].

One possible explanation for changes in condensed chromatin states affecting many chromosomal domains is different degrees of DNA methylation [8,17]. DNA methylation has been reported to be associated with carcinogenesis through various mechanisms [2,4,12,16,27]. In NIH 3T3 cells particularly, overexpression of cytosine (DNA-5)-methyltransferase leads to transformation [30]. In other cell systems, hypomethylation of proto-oncogenes and hypermethylation of tumor suppressor genes alter the expression levels of these genes, providing an epigenetic model for tumor development [12,27].

On the other hand, higher order chromatin organization (=supraorganization) is affected by nuclear matrix proteins [6,9,15,25], which might be advantageous to increase transcriptional efficiency and interactions facilitated by the proximity of active chromatin domains [26].

If DNA methylation were associated with chromatin supraorganization in the Ha-*ras*-transformed NIH 3T3 cell lines differing in *ras* expression and *ras*-induced metastatic ability, little variation in methylated DNA would occur since "condensed" chromatin areas and DNA values practically do not vary in these cell lines [19].

The participation of DNA methylation in "condensed" chromatin areas could be estimated *in situ* through video image analysis of Feulgen-DNA values after restriction enzyme treatment of these cells with either *Msp* I or *Hpa* II. *Msp* I and *Hpa* II are isoschizomeric restriction enzymes which cleave the base sequence -CCGG-, *Hpa* II being sensitive to methylation of the internal CpG dinucleotide [24]. In other words, *Hpa* II cuts the sequence CCGG but fails to cleave it if the central C is methylated. It has been demonstrated by digital image analysis that restriction enzymes may recognize, cleave and remove DNA from fixed chromosomes, giving rise to enzyme-specific images [10].

Although *Msp* I and *Hpa* II are considered useful tools for mitotic chromosome banding methods [23,28], they have not been applied up to now to whole interphase nucleus studies. Since the present investigation is centered on "condensed" chromatin areas and interphase heterochromatin is as condensed as are mitotic chromosomes [15,17], *Msp* I and *Hpa* II may also be suitable to interphase chromatin studies especially after acetic ethanol fixation (which extracts

many cell proteins), and extending digestion time for as long as the longest period proposed for mitotic chromosomes [23,28].

Here we determined by image analysis that the amount of methylated DNA in the "condensed" chromatin of interphase cell nuclei varied with the Ha-*ras*-transformed NIH 3T3 cell line, leading us to assume that methylated CpG sequences are not related to chromatin higher order condensation enhancement with *ras* transformation.

## 2. Materials and methods

### 2.1. Cells

A series of T24 Ha-*ras* oncogene-transformed NIH 3T3 cell lines previously isolated and characterized [7,11], as well as control NIH 3T3 cells, were grown in Dulbecco's modified Eagle medium (Grand Island Biological Co., Grand Island, New York, USA) with 10% calf serum (Grand Island). All the *ras*-transformed cells used are tumorigenic but differ in the levels of oncogenic *ras* protein expression and metastatic ability. The cell lines used were as follows:

C2P2, C5P2: lines derived from C2P0 and C5P0 clonal *ras*-transformed NIH 3T3 cells, respectively. C2P0 and C5P0 cells express very low levels of *ras* p21 protein, similar to those expressed by nontransformed NIH 3T3 cells, and are poorly metastatic, although tumorigenic. The C2P2 and C5P2 lines show increased metastatic ability in both chick embryos and nude mice and have higher proportions of *ras* protein expressing cells.

PAP2: cell line derived from PAP0 nonclonal, pooled population of *ras*-transformed NIH 3T3 cells, and selected *in vivo* for increased metastatic ability in both chick embryo and nude mice. PAP2 cells have a higher proportion of oncogenic *ras* protein-expressing cells compared to PAP0 cells.

NIH 3T3: nontumorigenic, nonmetastatic, control cells that express low levels of normal *ras* protein.

### 2.2. Cell preparations

Control and *ras*-transformed cells grown on coverslips were fixed in an absolute ethanol-acetic acid mixture (3:1) for one minute, rinsed in 70% ethanol for five minutes and air dried at room temperature.

### 2.3. Restriction enzymes

Digestions with *Msp* I and *Hpa* II from Amersham Pharmacia Biotech AB (Uppsala, Sweden) and Gibco BRL (Gaithersburg, MD, USA), respectively, were performed. Enzyme solutions were prepared according to conditions suggested by the supplier, at a final concentration of 0.5 U/ $\mu$ l. Coverslips of each cell line were covered with each enzyme solution. Incubations were carried out in a moist chamber at 37°C for 16 h [23, 28] and halted by rinsing the coverslips in stop buffer and assay buffer. Control preparations were incubated in the appropriate buffer without enzyme. Two sets of each cell line were used for each enzyme treatment and respective control.

### 2.4. Staining procedure

The preparations were subjected to the Feulgen reaction (4 N HCl at 25°C, 80 min; Schiff reagent, 1 h) and mounted in Canada balsam ( $n_D = 1.54$ ) [18].

### 2.5. Video image analysis

Zeiss-Kontron (Oberkochen-Munich, Germany) equipment was used for image acquisition, segmentation and featuring, by means of the Kontron-IPS system and appropriate programs according to the Kontron-IBAS 2000 manual of instructions [21,22].

The microscopic images were observed under a Zeiss (Oberkochen, Germany) Universal N microscope with a Neofluar 100/1.30 objective, optovar 1.25, 1.3 condenser, and  $\lambda = 565$  nm. The images to be processed were fed from the microscope into the computer through a monochrome CCD (Hamamatsu C3077) video camera. In this investigation, 1  $\mu$ m = 11.3 pixels.

A software macro developed by one of us (W.P.) provided quantitative morphological information on chromatin texture [21,22]. Different grey levels were assigned colors, for ease of display. After transformation of the original image, measurement inside the segmented object from the transformed image was carried out. Since densitometric features were derived from whole nuclei as well as from “condensed” chromatin regions, two segmentation levels were used. Although interactive image editing was possible during the run of the program, it was rarely required for the nuclei analyzed here.

Image analysis parameters pertinent to this study were:

1.  $A_T$ , total integrated absorbance (= nuclear Feulgen-DNA values);
2.  $A_c$ , integrated absorbance over a preselected absorbance threshold which was established for control nontransformed cell nuclei (type I nuclei) treated only with buffer. The same threshold level was automatically maintained for analysis of the nuclei with the other phenotypes and under enzyme treatment conditions.  $A_c$  corresponds to “condensed” chromatin Feulgen-DNA values; and
3.  $A_c\%$ , “condensed” chromatin Feulgen-DNA values relative to the nuclear (whole chromatin) Feulgen-DNA values.

Two hundred nuclei of each phenotype were measured for each cell line and experimental condition. One hundred nuclei were evaluated in each coverslip preparation. Data were analyzed statistically (descriptive statistics and nonparametrics) using the Minitab® statistical software for Windows (Minitab Inc., State College, PA, USA).

## 3. Results

### 3.1. Nuclear phenotypes

Nuclei from each cell type were characterized as previously described [19,20,22]. Briefly, the nuclear phenotypes observed are: I, I' – nuclei containing a few small granules of stained chromatin against a pale background; II – nuclei with abundant small granules of deeply stained chromatin; and III – nuclei with extensive areas of deeply stained coarse chromatin. Phenotypes I and II are present in nontransformed NIH 3T3 cells whereas phenotypes I' and III appear in the transformed cells. Nuclei with another phenotype, characterized by a few coarse and well-circumscribed chromatin granules against a very pale background (IV) and present at low frequency in the *ras*-transformed cells (<1.3% [19]), when detected, were added to the I' phenotype group due to their morphological similarity. Figure 1 shows examples of nuclei studied with the video image analysis system and photographed from the color monitor. Since “condensed” chromatin Feulgen-DNA relative values ( $A_c\%$ ) are the goal of present investigation, there was no preoccupation with selecting identical absolute nuclear sizes to show here.

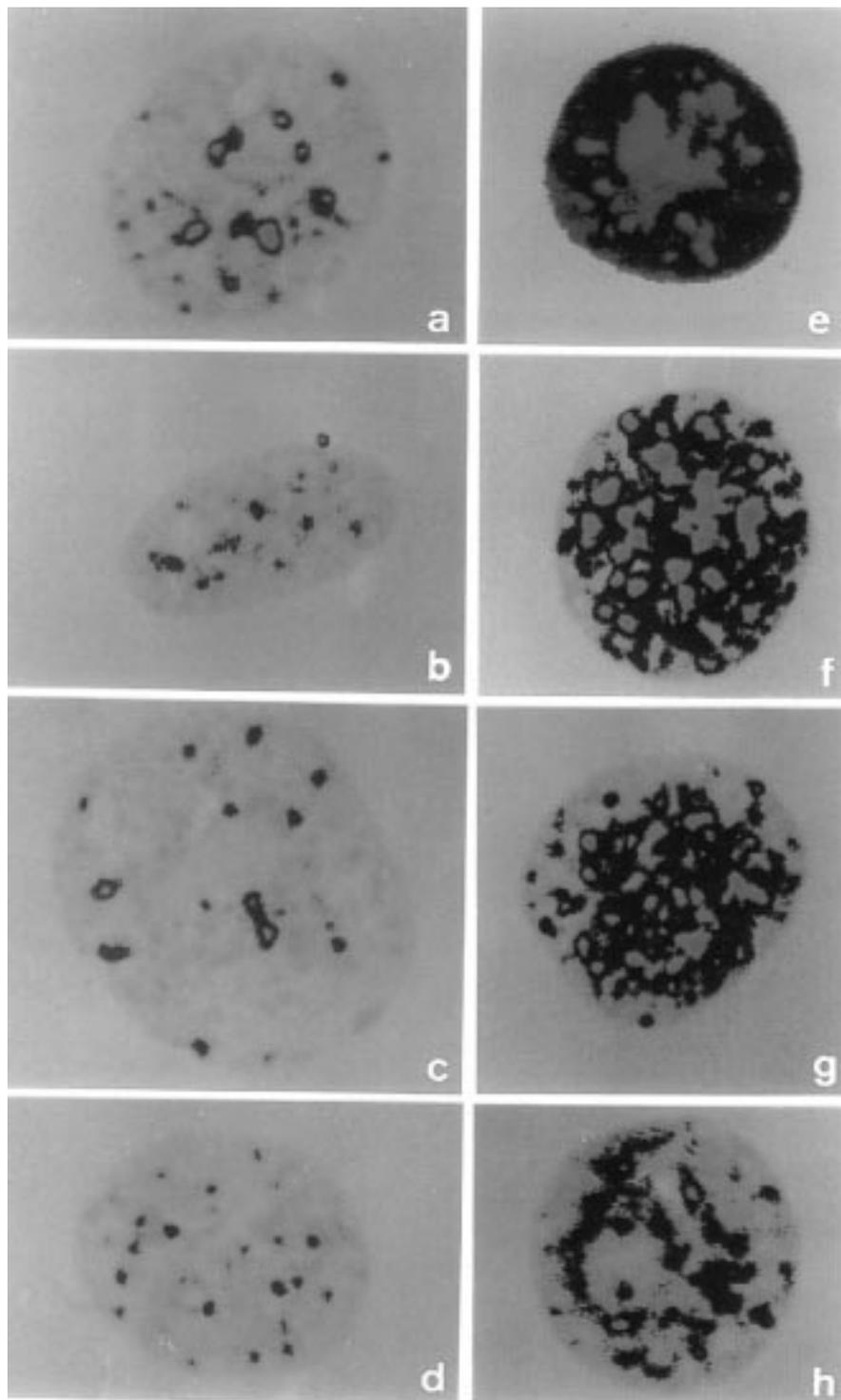


Fig. 1. Examples of pseudocolored images of Feulgen-stained type I nuclei (NIH 3T3 cells) (a–d) and type III nuclei (C5P2 cells) (e–h) subjected to *Msp* I (b, f) and *Hpa* II (d, h) treatments prior to staining, and photographed from the color monitor. Respective buffer-only controls, a, c, e, g.

### 3.2. Total Feulgen-DNA content

The distribution of the total nuclear Feulgen-DNA values ( $A_T$ ) for type II (III) relative to I (I') nuclei in nontransformed and *ras*-transformed cells after treatment with buffer alone (data not shown) was found to be in agreement with Mello and Chambers' [19] previous data for untreated cells. The Feulgen-DNA absolute values obtained for controls simply incubated in buffers differed from each other, when the two groups of assays were compared, a result attributed to different actions on chromatin of buffers which differed in composition [5]. For this reason, buffer-only treatment provides the appropriate control.

Total Feulgen-DNA content was not used for comparisons between buffer- and enzyme-treated cells since different ploidy degrees are found simultaneously in interphase nuclei of the different cell lines [19].

### 3.3. "Condensed" chromatin Feulgen-DNA content

$A_c\%$  values were used for comparisons since they were not affected by nuclear ploidy degrees [19]. The  $A_c\%$  values in the untreated *ras*-transformed cells analyzed here were found not to differ (Fig. 2). After *Msp* I treatment the  $A_c\%$  values for all cell types and nuclear phenotypes in comparison with buffer-only condition decreased (Fig. 3, Table 1). The values for buffer- and *Msp* I-treated type III nuclei of PAP2, C2P2 and C5P2 cells were found to differ significantly (Table 2). While the values for C2P2 and C5P2 cells compared to each other were found to differ in buffer- and *Msp* I-treated cells, the values for PAP2 cells differed from those of C5P2 cells in buffer-treated preparations but not in *Msp* I-treated cells (Table 2). Also the  $A_c\%$  values of *Msp* I-treated type I' nuclei of PAP2 and C5P2 cells were found not to differ (Table 2), although those of buffer-treated preparations differed (Fig. 3).

After the *Hpa* II treatment a decrease in  $A_c\%$  values was observed in all nontransformed NIH 3T3 cell nuclei and in type III nuclei of C5P2 cells. The other nuclear types did not show differences in  $A_c\%$  values after enzyme treatment or, in the case of type III nuclei of C2P2 cells and type I' nuclei of C5P2 cells, even larger values were exhibited (Fig. 4, Table 1). The  $A_c\%$  values of type I' and III nuclei of C2P2 cells compared to those of C5P2 cells were found not to differ in buffer-treated preparations (Table 3). When comparing the  $A_c\%$  values for *Hpa* II-treated C2P2 and C5P2 cells a difference was demonstrated only for type III nuclei

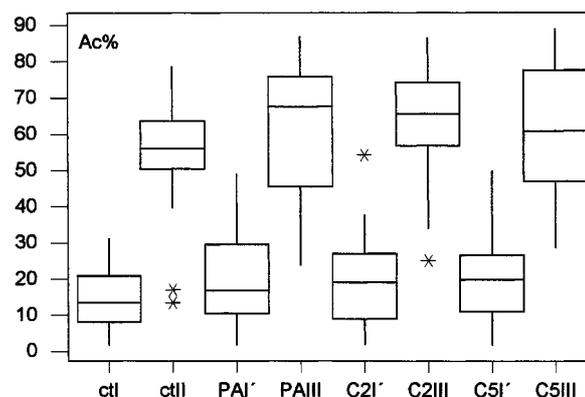


Fig. 2. Tukey's box-and-whisker plots (descriptive statistics, Minitab<sup>®</sup> statistical software) for "condensed" chromatin Feulgen-DNA relative values ( $A_c\%$ ) of nontransformed and *ras*-transformed NIH 3T3 cells. Lower and upper traces = lower and upper quartiles of the distributions; middle trace = median; \*, outliers; ct, control non-transformed cells; C2, C2P2 cells; C5, C5P2 cells; PA, PAP2 cells; I, I', II, and III, nuclear phenotypes.  $n = 200$ .

(Table 3). The  $A_c\%$  values of PAP2 and C5P2 type III nuclei, which differed under buffer treatment conditions, no longer differed after the *Hpa* II treatment, similarly as observed after the *Msp* I treatment (Tables 2, 3). Type I' nuclei of PAP2 and C5P2 cells were found not to differ from each other in either buffer- or *Hpa* II treatment conditions (Table 3).

## 4. Discussion

The results presented here indicate that methylated and unmethylated -CCGG- sequences occurring in the chromatin of NIH 3T3 cell lines treated with restriction enzymes could be determined when Feulgen-DNA values for "condensed" chromatin ( $A_c\%$ ) are studied *in situ* by image analysis.

The threshold level selected for consideration of "condensed" chromatin was the same used for discriminating this chromatin in untreated preparations of NIH 3T3 type I nuclei. It is reasonably comparable to a cut off point equal to absorbance 0.350 in a Zeiss automatic scanning microspectrophotometer. The morphological images of the "condensed" chromatin in NIH 3T3 type I nuclei under buffer-only condition as shown here (Fig. 1a,c) match well with those in Figs. 1a/1b and Fig. 1a from previously published reports for untreated cells ([21] and [22], respectively).

Since  $A_c\%$  values decreased with *Msp* I while remaining unchanged with *Hpa* II in nuclei with the same phenotype in some of the transformed cell lines,

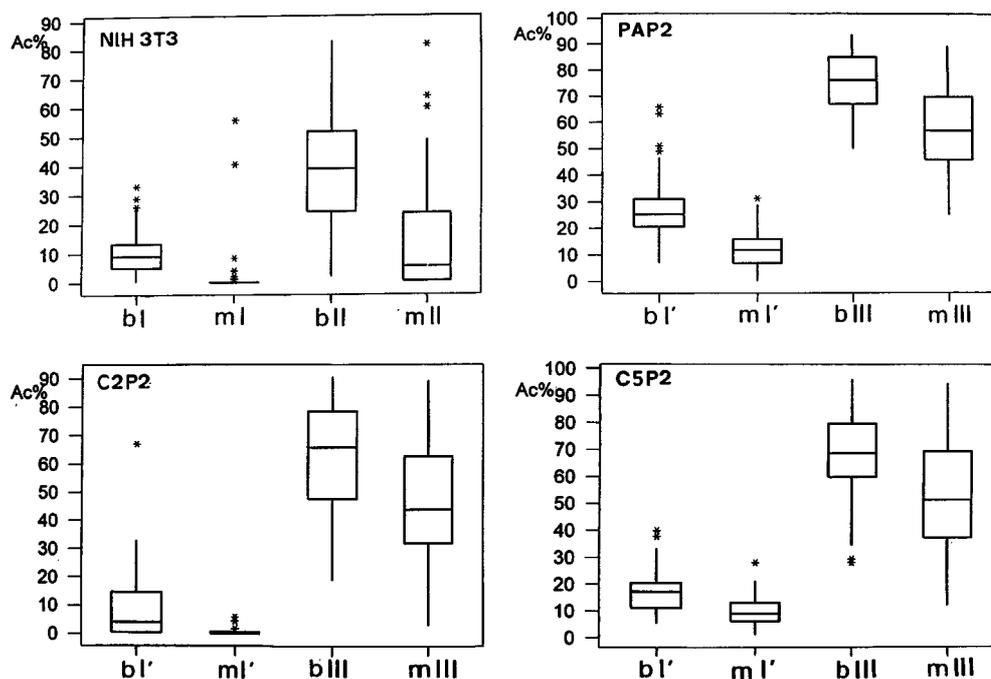


Fig. 3. Tukey's box-and-whisker plots (descriptive statistics, Minitab<sup>®</sup> statistical software) for "condensed" chromatin Feulgen-DNA relative values ( $A_c\%$ ) for nontransformed and *ras*-transformed NIH 3T3 cells. Preparations treated with *Msp* I (m) or just the *Msp* I buffer (b). Lower and upper traces = lower and upper quartiles of the distributions; middle trace = median; \*, outliers; I, I', II, and III, nuclear phenotypes.  $n = 200$ .

Table 1

Mann-Whitney test for "condensed" chromatin Feulgen-DNA content ( $A_c\%$ ) comparisons (Minitab<sup>®</sup> statistical software)

Cells	Nuclear phenotypes	Conditions compared	
		M ( <i>p</i> )	H ( <i>p</i> )
NIH 3T3	I	0.0000**	0.0000**
	II	0.0000**	0.0000**
PAP2	I'	0.0000**	0.1016
	III	0.0000**	0.1425
C2P2	I'	0.0000**	0.3804
	III	0.0000**	0.0000**
C5P2	I'	0.0000**	0.0032**
	III	0.0000**	0.0000**

H, *Hpa* II vs. respective buffer; M, *Msp* I vs. respective buffer; \*\*, highly significant ( $P_{0.01}$ );  $n = 200$ .

it is suggested that both methylated and unmethylated -CCGG- sites can co-exist in the "condensed" chromatin of the *ras*-transformed cells. If *Msp* I were unable to digest methylated CpG islands because intact nuclei were used here [1], *Msp* I and *Hpa* II digestion should give rise to similar results, which was not the case.

The variable decrease in  $A_c\%$  values with *Msp* I in the various NIH 3T3 cell lines indicates that the un-

methylated CpG sequences removed with this enzyme varied in amount with the cell line and the nuclear phenotype considered, being more abundant in the "condensed" chromatin of the nontransformed (control) cells. Methylated CpG sequences, on the other hand, were more frequent in the "condensed" chromatin of the *ras*-transformed cells but not equally abundant in the different cell lines.

Presence of unmethylated as well as of moderate to high levels of methylated CpG islands has been previously reported in nontransformed NIH 3T3 cells [1]. Hypermethylation in this case was considered to involve genes that mediate differentiation rather than genes essential for cell viability [1]. If *de novo* CpG methylation in transformed NIH 3T3 cells is confined to genes whose products are not essential under culture conditions, the variable response to *Msp* I/*Hpa* II enzymes in the "condensed" chromatin of different *ras*-transformed NIH 3T3 cell lines as found here may mean that different changes in the expression of genes required for survival were acquired with *ras* transformation.

It should be mentioned that  $A_c\%$  values of *Hpa* II buffer-treated nuclei were smaller than those of *Msp* I buffer-treated nuclei in *ras*-transformed cells. It is possible that the incubation in the assay buffers (espe-

Table 2

"Condensed" chromatin Feulgen-DNA content ( $A_c\%$ ) comparisons among *ras*-transformed nuclei (Minitab® statistical software)

Cells compared	Nuclear phenotypes	Treatment	Nonparametric tests	Conclusion
PAP2, C2P2, C5P2	III	buffer	Kruskal–Wallis	$H = 25.76$ , d.f. = 2, $p = 0.000$ (adjusted for ties)**
		<i>Msp</i> I	Kruskal–Wallis	$H = 16.54$ , d.f. = 2, $p = 0.000$ (adjusted for ties)**
C2P2, C5P2	III	buffer	Mann–Whitney	$p = 0.0488$
		<i>Msp</i> I	Mann–Whitney	$p = 0.0226^*$
PAP2, C5P2	I'	<i>Msp</i> I	Mann–Whitney	$p = 0.0527$
	III	buffer	Mann–Whitney	$p = 0.0006^{**}$
		<i>Msp</i> I	Mann–Whitney	$p = 0.0672$

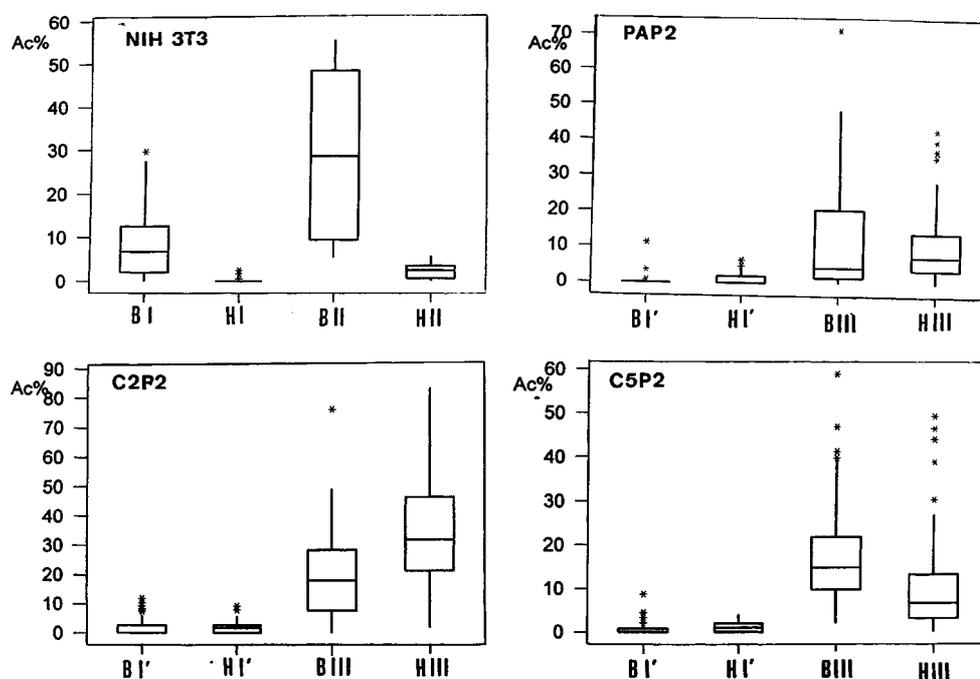
\*, significant ( $P_{0.05}$ ); \*\*, highly significant ( $P_{0.01}$ );  $n = 200$ .

Fig. 4. Tukey's box-and-whisker plots (descriptive statistics, Minitab® statistical software) for "condensed" chromatin Feulgen-DNA relative values ( $A_c\%$ ) for nontransformed and *ras*-transformed NIH 3T3 cells. Preparations treated with *Hpa* II (H) or just the *Hpa* II buffer (B). Lower and upper traces = lower and upper quartiles of the distributions; middle trace = median; \*, outliers; I, I', II, and III, nuclear phenotypes.  $n = 200$ .

cially, *Hpa* II buffer), induces some conformational change in the chromatin [5] causing earlier solubilization of part of the apurinic acid with Feulgen's acid hydrolysis [18]. When the assay buffer was supplemented with *Hpa* II, additional conformational changes might be introduced in the chromatin, delaying the above-mentioned step of the Feulgen kinetics. Such being the case, *Hpa* II assay buffer and enzyme actions would have been selective for the *ras*-transformed cells. Furthermore, this hypothesis may explain the increase

in  $A_c\%$  values for some nuclear types of the *ras*-transformed NIH 3T3 cells after *Hpa* II treatment.

The results obtained after the restriction enzyme treatments for the areas with increased chromatin higher order condensation varied in the different *ras*-transformed NIH 3T3 cell lines. Since the "condensed" chromatin areas and their corresponding DNA amounts differ little in the cell lines tested [19, Fig. 2], but do differ in their response to *Hpa* II treatment, it is assumed that CpG methylation may not be equally

Table 3

Mann–Whitney test for “condensed” chromatin Feulgen-DNA content ( $A_c\%$ ) comparisons (Minitab<sup>®</sup> statistical software)

Cells compared	Nuclear phenotypes	Treatment	Conclusion
C2P2, C5P2	I'	none	$p = 0.8659$
		buffer	$p = 0.1631$
		Hpa II	$p = 0.4957$
	III	none	$p = 0.4524$
		buffer	$p = 0.6868$
		Hpa II	$p = 0.0000^{**}$
PAP2, C5P2	I'	none	$p = 0.8795$
		buffer	$p = 0.1508$
		Hpa II	$p = 0.3454$
	III	none	$p = 0.6221$
		buffer	$p = 0.0000^{**}$
		Hpa II	$p = 0.4822$

<sup>\*\*</sup>, highly significant ( $P_{0.01}$ );  $n = 200$ .

present in the highly packed chromatin with *ras* transformation.

As a conclusion, it is suggested that methylated CpG DNA sequences are not associated with the chromatin condensation enhancement that accompanies *ras* transformation in NIH 3T3 cells, since the enhanced amount of methylated DNA detected in the “condensed” chromatin of these cells was found to vary with the cell line considered and not to accompany the invariable amount of chromatin condensation of the transformed cell lines. This idea is supported by a report demonstrating that DNA methylation is not involved with the structural organization at the molecular level of the ornithine decarboxylase gene and the bulk chromatin in c-Ha-*ras*<sup>(Val-12)</sup> oncogene-transformed NIH 3T3 cells [14].

## Acknowledgments

This study was supported by grants from the Brazilian National R & D Council (CNPq), the São Paulo State Research Support Foundation (FAPESP), the Alexander von Humboldt Foundation, the State University of Campinas Research Support Foundation (FAEP/UNICAMP), and the National Cancer Institute of Canada. The authors are indebted to Dr. Andrew H. Fischer (Emory University Hospital, Atlanta, USA) for criticism and helpful suggestions.

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