

## FEULGEN-DNA CONTENT EVALUATION AND IMAGE ANALYSIS OF NIH/3T3 CELLS TRANSFORMED BY MCF-7 CELL DNA

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### ABSTRACT

Feulgen-DNA amounts, nuclear sizes and phenotypes were microspectrophotometrically studied in NIH/3T3 cells transformed by transfection with MCF-7 cell *N-ras* oncogene-containing genomic DNA, and compared with those provided by a T24 *c-H-ras* DNA-mediated cell transformation. A situation characterized by increased levels of chromatin condensation affecting euchromatin was found, its general aspects resembling those generated by the T24 *c-H-ras* DNA-mediated transformation. The induced chromatin condensation is supposed to be associated with repression of genes rendered unavailable for transcription and/or a topological effect on nucleoprotein geometry promoted by neighboring or distant actions along the DNA. A condensation surpassing that demonstrated in the cells transformed by transfection with the T24 *c-H-ras* oncogene was detected in part of the cells transformed by transfection with the MCF-7 cell DNA. This is assumed to be due to a different clonal response of the NIH/3T3 cells to the transfected *ras* DNA or a consequence of transfection having been performed with the MCF-7 cell whole genomic DNA.

### INTRODUCTION

Three nuclear phenotypes have been demonstrated microspectrophotometrically and with image analysis for Feulgen-stained NIH/3T3 cells transformed by the *c-H-ras* oncogene of T24 cells. These phenotypes have been defined in terms of increased levels of chromatin condensation, differing from those of untransformed control cells (Mello and Russo, 1990).

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However, it has not yet been demonstrated whether the nuclear phenotypes found in the transformed cells were a specific response to T24 c-H-*ras* DNA transfection or an indiscriminated response to transfection with *ras* oncogenes.

It is well known that 3T3 cells are transformed by oncogenes of the *ras* family, including the N-*ras* oncogene of the MCF-7 human mammary carcinoma cell line (Becker *et al.*, 1984; Cooper and Lane, 1984; Barbacid, 1987; Burck *et al.*, 1988). This study thus aims at finding whether transformation of NIH/3T3 cells by a *ras* oncogene other than the c-H-*ras* one induces euchromatin condensation, giving rise to nuclear phenotypes such as those reported in the T24 c-H-*ras* DNA-mediated cell transformation (Mello and Russo, 1990). It is also part of an investigation on changes in chromatin supra-organizational states associated with cell transformation.

Feulgen-DNA content and nuclear image analysis were thus investigated in NIH/3T3 cells transformed after transfection with the N-*ras* oncogene-containing genomic DNA from MCF-7 cells. NIH/3T3 recipient cells were of the same origin as in the previously reported assays analyzing T24 c-H-*ras* DNA-mediated cell transformation (Mello and Russo, 1990).

## MATERIALS AND METHODS

### *Cell line*

NIH-3T3 cells obtained from Dr. M. Barbacid (Frederick Cancer Research Facilities, Maryland) and MCF-7 cells (clone E3) at passage 130 provided by Dr. B. Butler (Michigan Cancer Foundation) were used.

NIH/3T3 cells were cultured as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (Colorado Serum Co.). Cells were passaged when 90% confluent in dilutions ranging from 1:20 to 1:120. Incubators were maintained at 37°C, 10% CO<sub>2</sub> concentration and 100% humidity.

MCF-7 cells were grown in Eagle's minimal essential medium with Hank's salts (KC Biological) containing 5% calf serum in T25 culture flasks with a screw cap top in a 37°C incubator without CO<sub>2</sub>.

### *DNA transfection assay*

The recipient cells were NIH/3T3 cells at passage 9. Total genomic DNA of MCF-7 cells was used as the donor. Isolation of the MCF-7 cell DNA was done by the phenol extraction method of Perucho and co-workers (1981). The DNA transfer was done using a modification of Graham and van der Erb's (1973) calcium phosphate co-precipitation method (Wigler *et al.*, 1979). NIH/3T3 cells were incubated at 37°C in the presence of the precipitate, which was removed after 20-24 h. Foci ap-

peared 17-21 days post-treatment. NIH/3T3 cell plates were simultaneously cultured as controls.

### *Tumorigenic assay*

Fifty to fifty-five day-old nude mice (Balb/c nu/nu) were injected intramuscularly with  $10^6$  cells per animal. Controls were injected with untreated NIH/3T3 cells and experimentals received recultured foci of 3rd passage NIH/3T3 cells transfected with the DNA of the MCF-7 cells. All experimental mice injected with cells recultured from the foci grew tumors at two weeks post-injection, whereas mice injected with untreated NIH/3T3 cells grew no tumors. The tumors were removed from the mice under sterile conditions, minced with a scalpel in M199 solution, centrifuged, and digested with collagenase in a 37°C shaker bath overnight. The cells were cultured under the same conditions used for the NIH/3T3 cells. The first passage of cells obtained from the tumors was then analyzed.

### *Cell preparations*

Three sets of control and experimental assays were used. Slides of control NIH/3T3 cells and of the first passage of tumor cells were prepared after treatment of cell suspensions ( $1 \times 10^5$  cells/ml) with 0.05% trypsin in EDTA buffer, and then centrifuged for 5 minutes at 1250 rpm in a Shandon Cytospin 2 cytocentrifuge. The preparations were fixed in an ethanol - glacial acetic acid mixture (3:1) for one min, rinsed in 70% ethanol for five min, and air dried at room temperature.

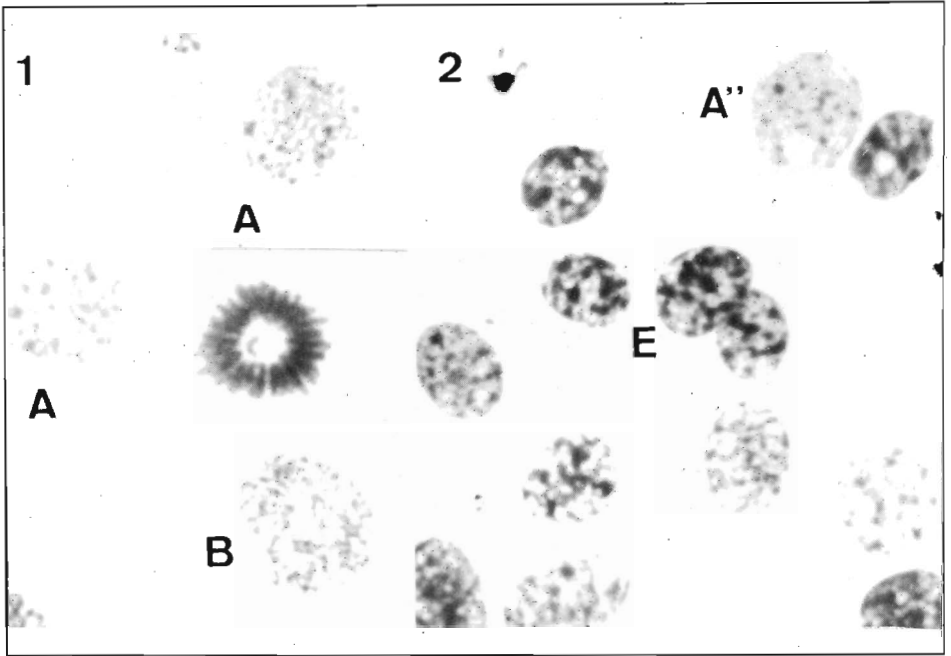
### *Staining reaction*

The preparations were subjected to the Feulgen reaction, the hydrolysis of which was carried out in 4M HCl at 25°C for 1 h and 30 min. The preparations were mounted in Canada balsam ( $n_D = 1.54$ ).

### *Nuclear phenotypes*

Microspectrophotometry was carried out for nuclei with phenotypes previously described for the NIH/3T3 cells and the NIH/3T3: MCF-7 tumor cells after a visual discrimination (Mello and Russo, 1989). These were: (A) Nuclei characterized by few small granules of stained chromatin on a pale background; (B) Nuclei exhibiting abundant small granules of stained chromatin; (A'') Nuclei with apparently the same staining aspects as A, but observed in the tumor cells; and (E) Nuclei with very conspicuous masses of condensed chromatin. The phenotypes A and B were detected

in control NIH/3T3 cells, whereas A'' and E phenotypes appeared in the tumor cells (Figures 1 and 2).



Figures 1 and 2 - Nuclear phenotypes of Feulgen-stained NIH/3T3 cells (1, A and B) and NIH/3T3:MCF-7 tumor cells (2, A'' and E) discriminated according to Mello and Russo's (1989) previous visual description. x 1050.

### *Scanning microspectrophotometry*

Feulgen-DNA values in arbitrary units and Feulgen-stained areas in  $\mu\text{m}^2$  were obtained with a Zeiss automatic scanning microspectrophotometer linked to a Microdata computer. Hardware and software were developed by Mr. Linus Vidal.

Operating conditions were: Planapo 100/1.25 objective, optovar 2, measuring diaphragm dia. = 0.1 mm, field diaphragm dia. = 0.2 mm, Zeiss LD-Epiplan 16/0.30 condenser,  $0.5 \mu\text{m} \times 0.5 \mu\text{m}$  scanning spot size and  $\lambda = 565 \text{ nm}$  obtained with a Schott monochromator filter ruler. The half band width at the point of transmission was quite small since the width of the effective light beam was equal to 0.2 mm (Zeiss Information, 1977). A predominantly monodirectional scanning motion was used. The grid points showing absorbances  $\leq 0.020$  were considered to be background and were automatically removed from the nuclear image.

*Image analysis*

The parameters evaluated were those used for the image analysis of the NIH/3T3 cells transformed with the c-H-*ras* oncogene of the T24 cells (Mello and Russo, 1990), based on previous reports by Vidal and his co-workers (1973, 1984). They were:

1. Total integrated absorbance ( $Abs_T$ ) = nuclear Feulgen-DNA values;
2. Nuclear stained area in  $\mu m^2$  ( $S_T$ );
3. Nuclear average absorbance ( $\bar{A} = Abs_T/S_T$ );
4. Integrated absorbance over a pre-selected absorbance value ("cut off" (c.o.) point) ( $Abs_C$ ) = "condensed" chromatin Feulgen-DNA values. Two c.o. point values were chosen, 0.200 and 0.300;
5. Area in  $\mu m^2$  covered by the stained chromatin discriminated after using the c.o. point ("condensed" chromatin) ( $S_C$ );
6. Area covered by the "condensed" chromatin relative to the nuclear stained area ( $S_C\%$ );
7. "Condensed" chromatin average absorbance ( $\bar{A}_C = Abs_C/S_C$ );
8. Average absorption ratio (AAR), which is the ratio of the "condensed" chromatin average absorbance to the entire nuclear average absorbance ( $AAR = \bar{A}_C/\bar{A}$ ).

**RESULTS**

The A" nuclei, which represent 7% of the tumor cell nuclear population (Mello and Russo, 1989), displayed Feulgen-DNA values concentrated in the same classes as the control A nuclei (89.4% of the control nuclear population (Mello and Russo, 1989)) (Figure 3). Some of the values for the A" tumor cell nuclei appeared slightly shifted to the left side of the Feulgen-DNA data distribution, when compared to the control. This could be promoted by aneuploidy in A" nuclei or by different phases of the cell cycle, when comparing A" nuclei to control A or B nuclei.

On the other hand, E nuclei, which are the most frequent nuclear phenotype of the tumor cells (Mello and Russo, 1989), exhibited Feulgen-DNA values distributed in the same classes as A and B nuclei, but the distributional pattern of their Feulgen-DNA content rather resembled that of B nuclei (Figure 3).

Considering that the metaphase plates of the NIH/3T3 control cells exhibited a 4C DNA content (Figure 3), it is assumed that the interphase nuclei of control and tumor cell populations with the same DNA content may be undergoing the DNA replication which precedes the next cell division.

Though not very frequently, polyploidy was also found in E nuclei and in control B nuclei (Figure 3).

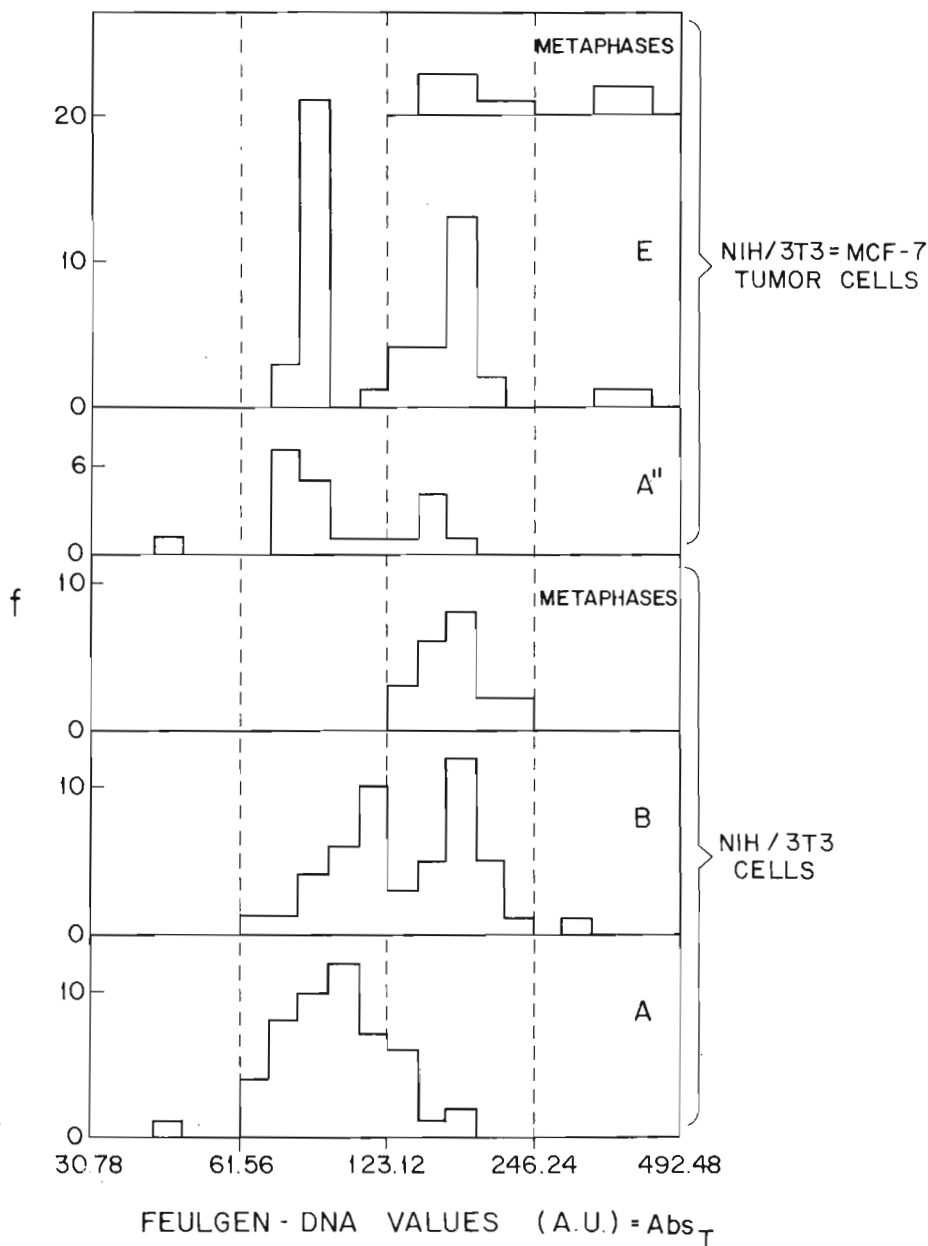


Figure 3 - Feulgen-DNA values ( $Abs_T$ ) in arbitrary units (A.U.) for nuclei with phenotypes A, B (NIH/3T3 control cells), A'' and E (NIH/3T3:MCF-7 tumor cells). Some metaphase values were also plotted. The dashed lines correspond to Feulgen-DNA content intervals with an increasing ratio of 2.  $f$  = frequency.

The nuclear absorbing areas appeared markedly decreased in the tumor cell population compared to the control (Figure 4). This has also been verified in toluidine blue-stained preparations (Mello and Russo, 1989).

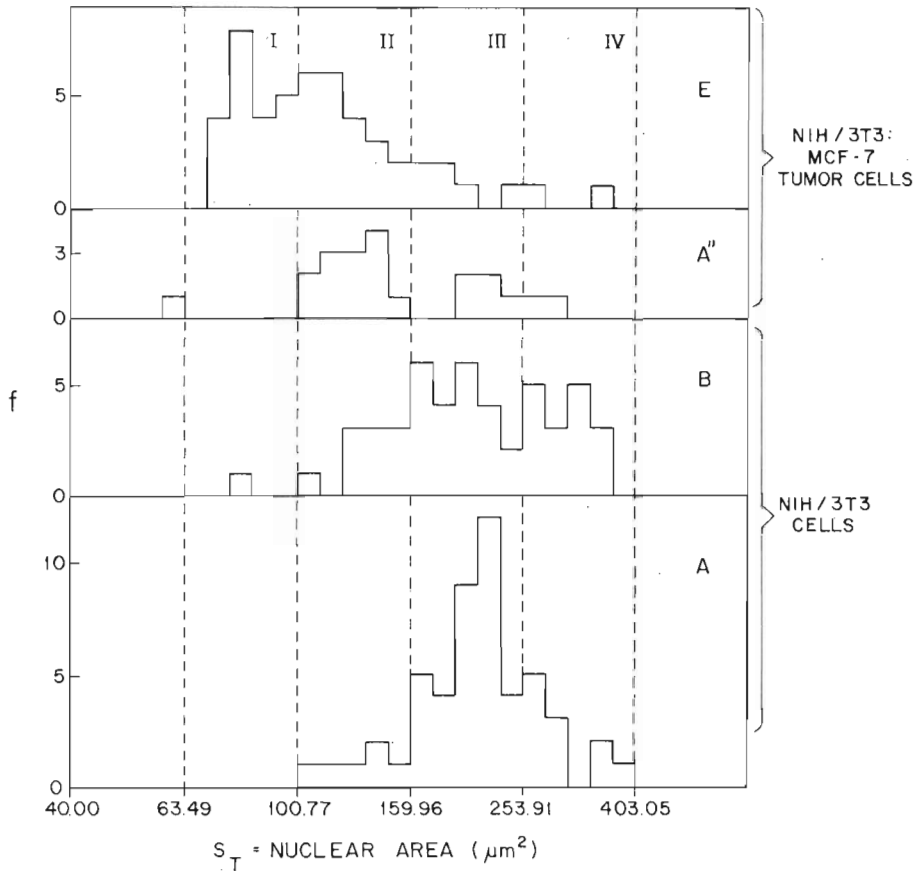


Figure 4 - Nuclear areas for the Feulgen-stained control (nuclear phenotypes A and B) and tumor cells (nuclear phenotypes A'' and E). I to IV =  $S_T$  intervals with an increasing ratio of  $^3/4$ , which corresponds to a doubling of nuclear volume (Palkóvits and Fischer, 1968);  $f$  = frequency.

The relationship between  $Ab_{S_T}$  and  $S_T$  values is shown in the scatter diagram in Figure 5. From this diagram it can be deduced that most tumor cell E nuclei, though presenting Feulgen-DNA amounts ( $Ab_{S_T}$ ) differing little from those of control A and B nuclei, exhibited much smaller areas ( $S_T$ ). In addition, most A'' nuclei, which exhibited a Feulgen-DNA amount as small as that of the smaller control nuclei, presented areas equal to or smaller than those of A and B nuclei.

Table I was prepared with data obtained after varying discrimination of the Feulgen-stained areas covered by condensed chromatin. This was carried out using

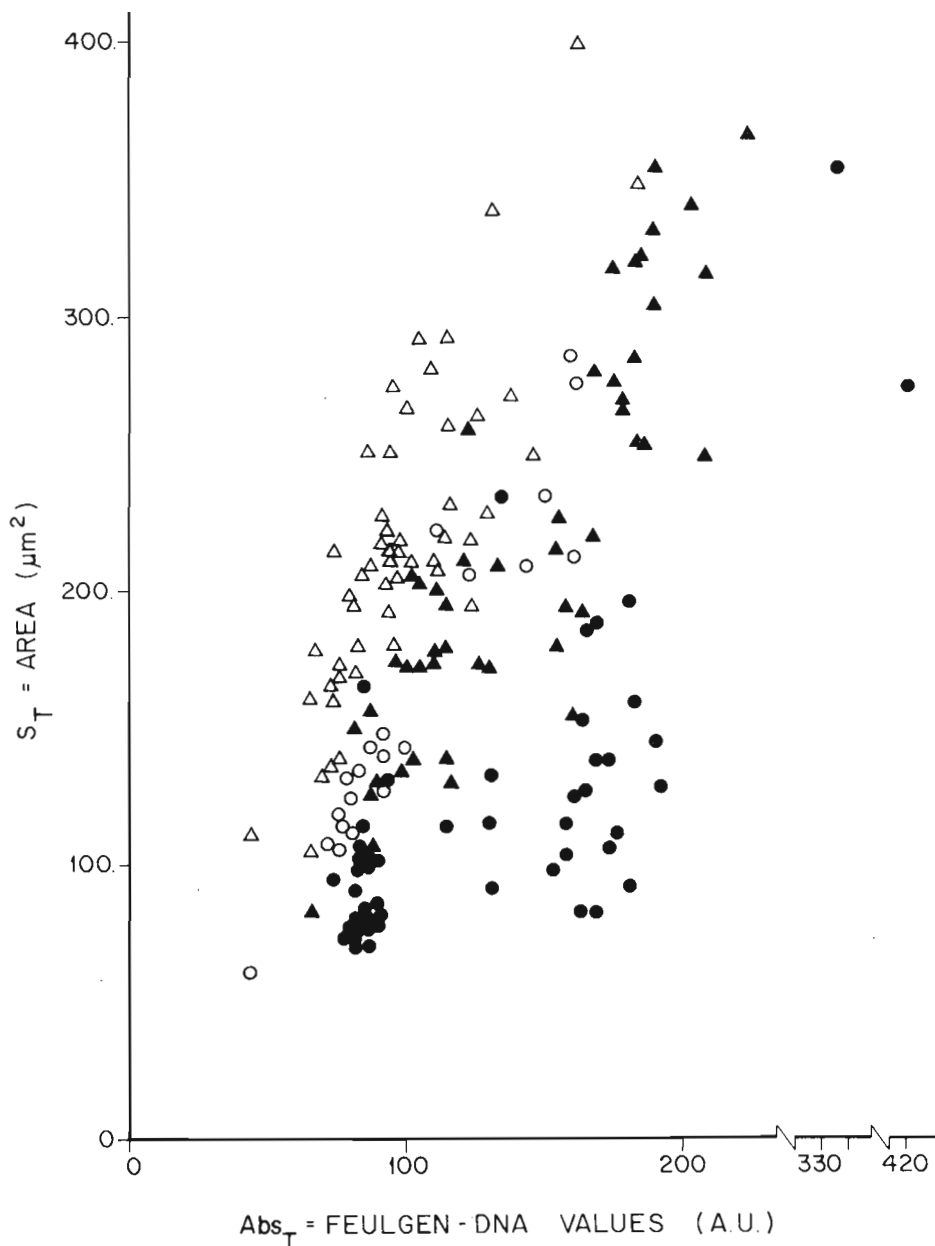


Figure 5 - Relationship between Feulgen-DNA values ( $\text{Abs}_T$ ) and nuclear areas ( $S_T$ ). Phenotypes A ( $\Delta$ ) and B ( $\blacktriangle$ ) = NIH/3T3 cells. Phenotypes A'' ( $\circ$ ) and E ( $\bullet$ ) = NIH/3T3:MCF-7 tumor cells. A.U. = arbitrary units.

a c.o. point equal to 0.200, which under present experimental conditions discriminates the granules of condensed chromatin [heterochromatin (Pardue and Gall, 1970)] of

control A nuclei, and a c.o. point equal to 0.300, which has proven to be the most adequate to discriminate the various nuclear phenotypes found in the NIH/3T3 cell assay after a T24 c-H-*ras* DNA-mediated transformation (Mello and Russo, 1990).

Table I - Feulgen-stained areas ( $S_C$ ) and Feulgen-DNA amounts ( $Abs_C$ ) discriminated at different c.o. points for NIH/3T3 cell nuclei.

c.o. points	Parameters	Interval	Frequency (%) of nuclei				
			Control	Cells	Tumor	Cells	
			A n = 51	B n = 49	A" n = 21	E n = 50	
0.200	$S_C$ ( $\mu\text{m}^2$ )	< 0.25	5.9	-	-	-	
		0.25 - 30.	84.3	-	14.3	2.0	
		30.25 - 50.	9.8	22.4	52.4	40.0	
		50.25 - 110.	-	63.3	33.3	54.0	
		110.25 - 170.	-	14.3	-	4.0	
	$S_C$ (%)	0 - 10.	76.4	-	-	-	
		10.1 - 30.	23.6	36.8	33.3	4.0	
		30.1 - 70.	-	63.2	66.6	70.0	
		70.1 - 100	-	-	-	26.0	
	$Abs_C$ (%)	0 - 20.	78.5	-	-	-	
		20.1 - 40.	21.5	16.4	19.0	4.0	
		40.1 - 70.	-	67.3	80.9	12.0	
		70.1 - 100	-	16.3	-	84.0	
	0.300	$S_C$ ( $\mu\text{m}^2$ )	< 0.25	49.0	-	-	-
			0.25 - 10.	51.0	42.9	47.6	2.0
			10.25 - 30.	-	44.9	52.4	34.0
30.25 - 60.			-	12.2	-	34.0	
60.25 - 140.			-	-	-	30.0	
$S_C$ (%)		0 - 5.	100	38.8	28.6	2.0	
		5.1 - 40.	-	61.2	71.4	48.0	
		40.1 - 100	-	-	-	50.0	
$Abs_C$ (%)		0 - 5.	96.0	24.5	9.5	-	
		5.1 - 30.	4.0	63.3	90.5	8.0	
		30.1 - 100	-	12.2	-	92.0	

n = number of measurements; - = zero.

The condensed chromatin stained regions varied either in size and percentage of the nuclei they occupied or in Feulgen-DNA amounts, according to the nuclear phenotype considered.

When an absorbance value of 0.200 was used as the c.o. point, chromatin regions discriminated in the B, A'' and E phenotypes were larger than those distinguished for A nuclei and did not exclusively discern heterochromatin. A'' nuclei, though visually undistinguishable from A nuclei, could attain 30-50% of their area ( $S_C$ ), displaying absorbances higher than 0.200, a situation never attained by nuclei with the A phenotype (Table I).

When the c.o. point level was elevated to 0.300 all A'' nuclei but only half of control A nuclei showed distinguishable areas ( $S_C$ ). The discriminated areas did not exceed 5% of the whole absorbing area in A nuclei, but attained up to 28% of the whole area in A'' nuclei. Most E nuclei displayed  $S_C$  and  $S_C\%$  values larger than those of A'' nuclei. On the other hand, though the  $S_C$  values of most E nuclei are equal to those of B nuclei, the  $S_C\%$  of the former were found to be larger than those of the latter (Table I).

The Feulgen-DNA values of the "condensed" chromatin ( $Abs_C$ ) in most A nuclei represent less than 20% of the entire nuclear DNA content, when discerned at the c.o. point 0.200, and less than 5%, when discriminated at the c.o. point 0.300. On the other hand, in most B nuclei the  $Abs_C$  contribute about 40 to 80% of the entire nuclear Feulgen-DNA content at the c.o. point 0.200, but generally up to 30% at the c.o. point 0.300. In the tumor cell nuclei, the regions of "condensed" chromatin of the A'' nuclei contained much more Feulgen-DNA than those of control A nuclei and the "condensed" chromatin zones of the E nuclei showed the most Feulgen-DNA (Table I).

The four nuclear phenotypes described in this investigation could be discriminated among themselves when plotting AAR values vs  $S_C$  (%), especially at the 0.300 c.o. level (Figure 6).

## DISCUSSION

Present results indicate that a change in patterns of chromatin condensation, also affecting nuclear areas, occurs in interphase nuclei of NIH/3T3 cells transformed by genomic DNA of MCF-7 cells. Nuclear phenotypes with increased levels of chromatin condensation could be detected, a situation which in its general aspects resembles that of NIH/3T3 tumor cells generated by the T24 c-H-ras DNA-mediated transformation (Mello and Russo, 1990). Even A'' tumor cell nuclei, containing few small granules of deeply stained chromatin on a pale background, and visually resembling control A nuclei could be demonstrated, through image analysis, to have their condensed chromatin areas enlarged, compared to A nuclei. The points of condensed

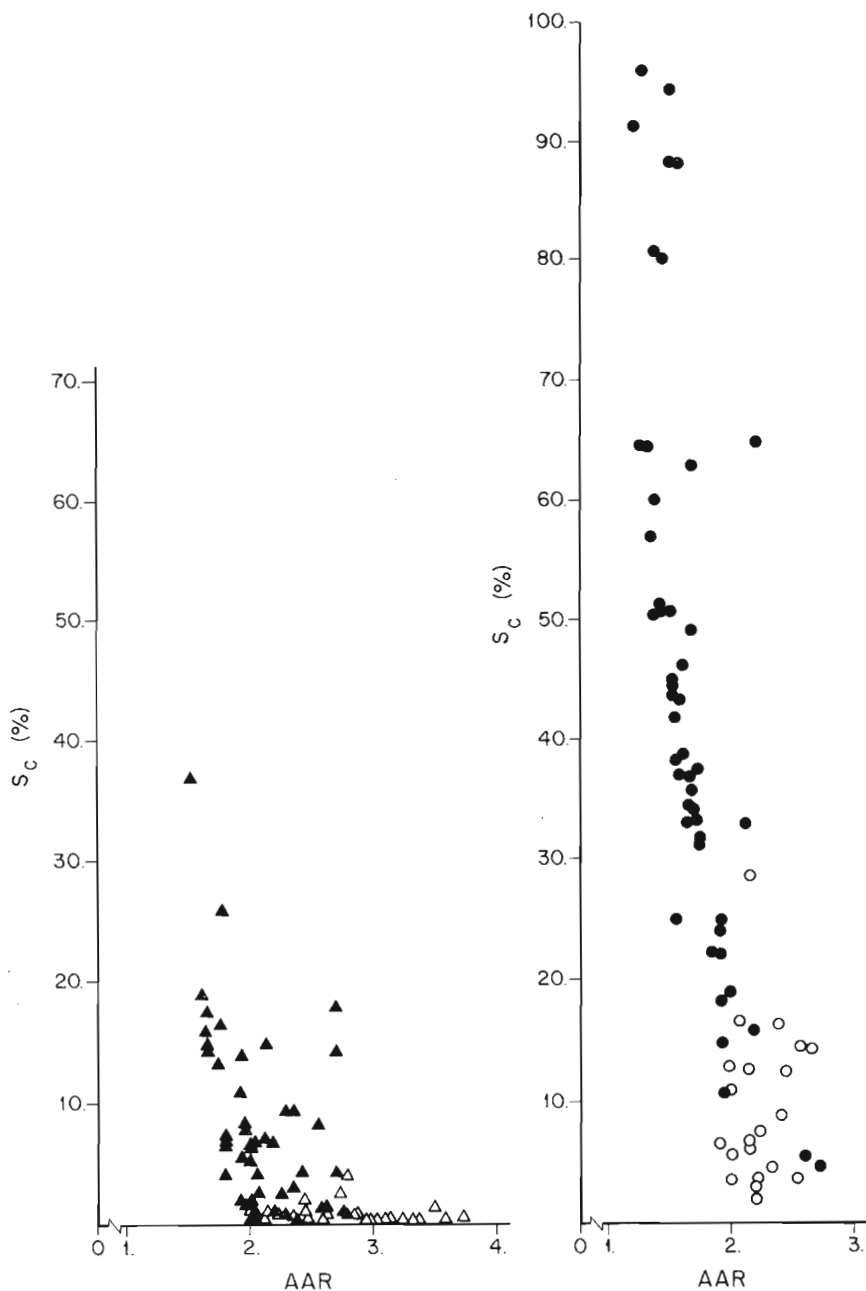


Figure 6 - Relationship between AAR (see text) and "condensed" chromatin area ( $S_C\%$ ) for NIH/3T3 cells with the nuclear phenotypes A ( $\Delta$ ) and B ( $\blacktriangle$ ) and for NIH/3T3:MCF-7 tumor cells with the phenotypes A' ( $\circ$ ) and E ( $\bullet$ ). The c.o. point was equal to 0.300.

chromatin discriminated at c.o. point 0.200 in control A nuclei under present experimental conditions represent heterochromatic areas (Mello and Russo, 1990). The chromatin condensation demonstrated under conditions of MCF-7 DNA-mediated transformation, like that of T24 c-H-*ras* DNA-mediated transformation, affects euchromatin (Mello and Russo, 1990).

Thus, chromatin condensation in NIH/3T3 cells is not indicative of induction by a specific *ras*-oncogene DNA-mediated transformation. According to Nicolini and his co-workers (1982), a limited transcription of the genome associated with nuclear chromatin condensation is required to maintain the transformed phenotypes. The euchromatin condensation exhibited by the transformed cells may thus be related to repression of genes rendered unavailable for transcription. It may also be a topological effect at the supra-nucleoprotein structure level through influence of multiple neighboring actions or at a distance along the DNA (Wang and Giaever, 1988).

A few differences in patterns of chromatin condensation and nuclear areas could be detected after comparing the situations involving MCF-7 DNA- and T24 c-H-*ras* DNA-mediated transformations with each other. These differences refer to: 1. nuclear areas ( $S_T$ ) for the part of the cell nuclei with coarser chromatin granules, 2.  $S_C$  for more densely stained chromatin (discrimination at the larger c.o. point), and 3.  $S_C$  and  $S_C\%$ , AAR values and AAR vs.  $S_C\%$  relationship for nuclei with small conspicuous granules on a pale background.

The data summarized in Table II lead to the conclusion that when NIH/3T3 cell transfection was carried out by MCF-7 cell DNA, a condensation surpassing that expected from data obtained for transformation mediated by T24 c-H-*ras* DNA transfection may affect the loosely packed chromatin for part of the transformed cell nuclei, irrespective of their described phenotypes. This excessive condensation may represent a different clonal response of the recipient cells to the transfected *ras* DNA (Mello and Chambers, 1990) or it could have been promoted by the different composition of the exogenous DNA, which in fact was not only that of an isolated N-*ras* oncogene, but of the whole genome of MCF-7 cells.

The chromatin condensation found in the NIH/3T3 cells after the DNA-mediated transformation is not supposed to have been contributed by a "spontaneous" transformation, as this is characterized by nuclear phenotypical changes of another magnitude (Mello, 1990).

Though polyploidy has been reported not to be an essential step in murine transformation (Miller and Miller, 1983), some few polyploid nuclei were found in control (B phenotype) and tumor (E phenotype) cells. For the NIH/3T3 cells transformed by transfection with the T24 c-H-*ras* oncogene, polyploidy has been verified preferentially in D nuclei, defined as those with very large clumps of condensed chromatin on a moderately stained background and representing only 3.2% of the transformed nuclear population (Mello and Russo, 1989, 1990). This phenotype has not been verified in the tumor cells analyzed in this investigation.

Table II - Differences discriminated after a comparative image analysis was carried out for NIH/3T3:T24c-H-ras tumor cells vs. NIH/3T3:MCF-7 DNA tumor cells.

Parameters	Nuclei compared	Observations	Conclusions
St	C* x E**	some C nuclei much smaller than E nuclei	Aneuploidy possibly much more evident in nuclei with coarse chromatin granules in cells transformed by transfection with the c-H-ras oncogene
Sc (%) (c.o. = 0.300)	C* and D* x E**	C nuclei: 5-60% D nuclei: 20-80% E nuclei: 0-100% 16% of E nuclei > 80%	Enhanced chromatin condensation extending to nearly the entire nuclear chromatin affects part of the nuclei, with coarse chromatin granules only in cells transformed by transfection with MCF-7 cell DNA
Sc (c.o. = 0.200)	A'' x A''**	most A' nuclei: 0.25-40 $\mu\text{m}^2$ A'' nuclei: 20.25 - 90 $\mu\text{m}^2$ 24% of A'' nuclei: 70-90 $\mu\text{m}^2$	
Sc (%) (c.o. = 0.200)	A'' x A''**	19% of A' nuclei and 63% of A'' nuclei; Sc (%) = 30-50	Part of the loosely packed chromatin undergoes a moderate condensation in a large % of nuclei showing the phenotype
AAR (c.o. = 0.300)	A'' x A''**	A' nuclei: up to value 5; most data between values 2. and 4 A'' nuclei: up to value 3; most data between values 2. and 3.	"few and small densely stained chromatin granules on a pale background" in the cells transformed by transfection with MCF-7 cell DNA
AAR x Sc (%) (c.o. = 0.300)	A'' x A''**	different distribution of correlated data	Phenotypes A' and A'' can be discriminated graphically

\* = NIH/3T3: T24 c-H-ras tumor cells (Mello and Russo, 1990); \*\* = NIH/3T3: MCF-7 DNA tumor cells.

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## RESUMO

O conteúdo Feulgen-DNA, bem como os tamanhos e fenótipos nucleares de células NIH/3T3 transformadas por transfecção com o DNA genômico contendo oncogene *N-ras* de células MCF-7, foram estudados por microespectrofotometria de varredura e análise de imagem e comparados aos exibidos quando a transformação é obtida por transfecção com o DNA do oncogene *c-H-ras* de células T24. Foram encontrados níveis superiores de condensação cromatínica afetando a eucromatina, o que em seus aspectos gerais caracteriza uma situação semelhante àquela obtida quando a transformação é mediada pelo DNA do oncogene *c-H-ras* de células T24. Supõe-se que a condensação cromatínica induzida esteja associada a uma repressão de genes tornados não disponíveis para transcrição e/ou represente um efeito topológico sobre a geometria de complexos nucleoproteicos, induzido por ações próximas ou distantes ao longo da molécula de DNA. Em parte das células transformadas pela transfecção com o DNA das células MCF-7 a condensação cromatínica excedeu aquela das células transformadas por transfecção com o oncogene *c-H-ras*. Admite-se que isto seja uma diferente resposta clonal de células NIH/3T3 após transfecção com oncogenes *ras* ou seja causado pela composição do DNA transfectado, o qual compreendia o genoma total de células MCF-7.

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