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Competence Growth Factors Can Cause Modification in Higher-Order Chromatin Structure in Mouse Embryo 3T3 Fibroblasts

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The authors compared sedimentation rates of nucleoids from mouse embryo 3T3 fibroblasts cultured in the presence or absence of different cell growth factors. The results clearly showed that rapidly sedimenting nucleoids are obtained only when cells are supplied with any of the following competence growth factors: platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), or the product of the oncogene v-sis (a peptide homologous to PDGF). The tumor promoter phorbol 12-myristate 13-acetate, an activator of protein kinase C and a partial mitogen, shares this property with the competence growth factors. Removal of these factors from the medium causes cells to enter Go and nucleoids to sediment at a slower rate. Protein synthesis is required for growth factor induction of change in nucleoid sedimentation, but inhibition of either DNA synthesis or DNA repair does not antagonize the effect of growth factors. Titration of nucleoids with ethidium bromide indicates that one possible mechanism for the nucleoid change is the unwinding of DNA in supercoiled loops. The results indicated that the nucleoid change constitutes a cell response to competence factors that might have an important role in cell proliferation.

Key words: cell cycle, PDGF, FGF, PMA

The 30 nm interphase chromosome fibers are arranged into supercoiled loops, each containing approximately 100 kbp of DNA [1–3]. The torsional constraints on the DNA on each loop seem to be provided by components of the nuclear matrix [3]. This higher order of organization of the chromosome has been implicated in the modulation of DNA replication [4] and transcription [5]. On the other hand, it has been clearly demonstrated that cell growth factors perform their control activity through the establishment of a hierarchical program of transcription [6]. Several laboratories have focused attention on

Abbreviations: ara-cyt, arabinosylcytosine; ddT, dideoxythymidine; EtdBr, ethidium bromide; FCS, fetal calf serum, FGF, fibroblast growth factor; PDGF, platelet derived growth factor; PMA, phorbol 12-myristate 13-acetate; thymidine, dT.

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changes in chromatin structure, either induced in Go cells by growth factors or during cellular differentiation [7]. Evidence has been obtained that a higher-order chromatin structure is lost during differentiation of mouse neuroblastoma cells and in nondividing cells [7]. It has also been observed that the number of topological ("titratable superhelical") turns in closed superhelical loops of nuclear DNA decreases in a first step of Friend erytroleukemia cells differentiation and during senescence of Syriam hamster cells [8,9]. Supporting these findings is the recent report that topoisomerase II activity is increased when mouse 3T3 cells pass from quiescence to proliferation [10]. As a consequence of these lines of evidence, it is expected that a change in higher-order chromatin structure would occur when cycling cells were induced to quiescence (Go). The authors decided to characterize such chromatin alteration in 3T3 cells, a very useful in vitro model for the investigation of cell cycle control [11]. When deprived of growth factors, these cells enter a quiescent state called Go. These factors are peptides, which fall into two categories, namely, competence and progression factors. Competence factors, such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), render Go cells competent to respond to progression factors (such as epidermal growth factor and insulin-like growth factor) and thus to progress to S-phase and cell division [12].

To investigate alterations of the supercoiled loops, the nucleoid sedimentation analysis technique was used. Nucleoids consist of nuclear supercoiled DNA, depleted of most proteins, which is constrained into loops by attachment to a residual nuclear matrix [1,2]. Changes in nucleoid sedimentation rate have been observed in different situations: 1) DNA breakage, either upon treatment with genotoxic agents [13] or upon differentiation [14,15]; 2) association of proteins to the nuclear matrix in heat-shocked cells [16]; 3) decrease of DNA superhelical turns in differentiated and senescent cells [8,9]; 4) decrease of DNA loop size in S-phase cells [17] and an increase in DNA loop and replicon sizes during ontogenesis [4]. The major problem of this technique is the difficulty in determining among these possibilities which one is responsible for a change in nucleoid sedimentation. Using this procedure, cell growth factors were found to have a fundamental role in the transition of the nucleoid structure from a slow to a rapid sedimenting state, while simultaneously causing cells to pass from Go to proliferation.

MATERIALS AND METHODS

Cells and Cell Treatment

Swiss 3T3 mouse embryo fibroblasts [18], having undergone few passages in culture, were obtained from Dr. Hugo A. Armelin's laboratory (University of Sao Paulo). The cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS), 472 units ml^{-1} penicillin, and 97 ug ml^{-1} streptomycin, in a 5% CO_2 humidified atmosphere at 37°C. In some experiments, the medium was supplemented with 0.2% FCS or 5% human plasma instead of 10% FCS. The following additions were tested for their effect on chromatin structure and cell proliferation: 3-aminobenzamide, cycloheximide, arabinosylcytosine (ara-C), dideoxythymidine (ddT), and phorbol 12-myristate 13-acetate (PMA) (Sigma). FGF was a kind gift from Dr. A.G. Gambarini (University of Sao Paulo); it corresponded to fraction 1 M from carboxymethyl-Sephadex [19]. One unit of FGF is defined as the volume of the preparation required to induce [^{3}H]-thymidine incorporation in an amount corresponding to 50% of that induced by 5% FCS medium in sparse Swiss 3T3 cells. Human plasma

and platelet extract were kindly provided by Dr. Mari C.S. Armelin (University of Sao Paulo). The latter was used as a source of PDGF [20]. One unit of PDGF activity is the volume (ul) of this extract required to induce 50% of resting confluent Balb cells to enter the S-phase. In some experiments, the 0.2% serum medium was conditioned for 48 h with confluent normal rat kidney (NRK) cells, which are transformed by the simian sarcoma retrovirus and secrete the gene product of the oncogene v-sis [21] homologous to PDGF II [22,23].

Nucleoid Sedimentation

The protocol described by Cook and Brazell [24] was followed with some modifications [25]. Cells were prelabeled for 2 days either with 0.05 uCi ml^{-1} [^{14}C]-thymidine or with 0.2 uCi ml^{-1} [^{3}H]-thymidine. After reaching confluence, they were maintained in medium with 10% serum for 2–3 days before being submitted to the treatment indicated. At the prescribed times, the cells were trypsinized and resuspended in 3 ml phosphate-buffered saline. After centrifugation for 15 min at 600 g at 4°C, the cells were resuspended in the same buffer (300–600 $\times$ 10^3 cells/100 ul), and 50 ul of this suspension was added to 250 ul of lysis solution containing 2.28 M NaCl, 24 mM EDTA, 0.6% Triton X-100, and 10 mM Tris-HCl, pH 8.0. The mixture was layered over 4.5 ml of a 15–30% sucrose gradient containing 1.9 M NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 8.0, and left to stand for 15 min before centrifugation at 20°C for 30 min at 9,000 rpm in a SW 50.1 Beckman rotor. Fractions (0.2 ml) were collected on strips of Whatman paper 17. After drying, the paper strips were cut, placed in vials with 5.0 ml toluene containing 4 g/liter 2,5-diphenyloxazole, and 0.1 g/liter p-bis (2-(5-phenyloxazolil))-benzene, and their radioactivity was counted in a scintillation spectrometer. Sedimentation rates were determined by taking as a reference the interpolated fraction, which divides the peak in two equal halves. In some experiments, varying concentrations of ethidium bromide (0–25 ug/ml) were present in the gradient.

Protein Synthesis

To the medium 5 uCi ml^{-1} [^{3}H]-leucine were added, and incubation was allowed to proceed for 2 h. The cells were rinsed with phosphate-buffered saline, 5% trichloroacetic acid (4°C), and 95% ethanol and lysed with 0.3 M NaOH; 200 ul aliquots were placed onto pieces of Whatman 17 paper. After drying, the samples were placed in vials with 5 ml of scintillation liquid and the ^{3}H counts were determined in a scintillation spectrometer. Absorbance at 260 nm of the NaOH lysate was measured, and the ^{3}H cpm/A_{260} ratio was taken as the rate of protein synthesis.

RESULTS

Most of the results presented here show a difference in chromatin structure between cells maintained in 10% serum medium and cells incubated in 0.2% serum medium, as measured by the nucleoid technique. Figure 1 illustrates the typical profiles of nucleoids from confluent cells incubated with 10% serum (peaks on the left) or 0.2% serum (peaks on the right). The results are in duplicate and show that, although not large, the difference in migration rates is significant. In this experiment, the two nucleoid peaks from cells incubated in 0.2% serum migrated 81% and 79% in relation to the peaks from control cells.

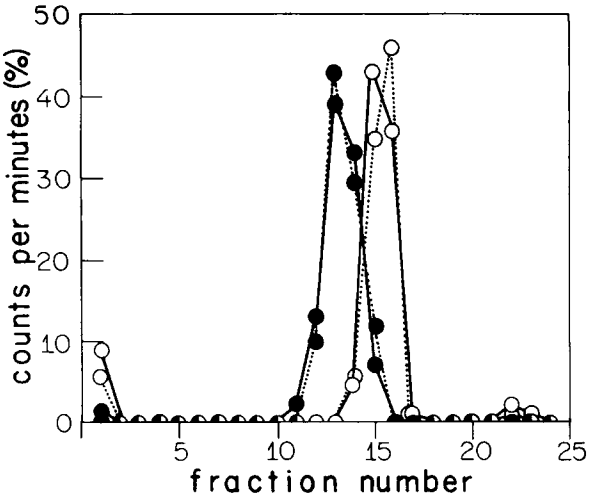

Fig. 1. Typical profiles of nucleoids from cells incubated with 10% serum or 0.2% serum. [^{3}H]-thymidine-labeled confluent cells were supplied with 10% serum (●) or 0.2% serum (○) for 1 day. Nucleoids were prepared and sedimented as described in Methods. Sedimentation is from the right to the left. Duplicates are depicted by different lines (——, -----).

Figure 2 shows that the presence of factor(s) in serum is important to maintain nucleoids in a fast sedimentation state. With the exception of the experiment in Fig. 5, in all the experiments with nucleoids, the cells were radioactively labeled during the exponential growth phase and maintained in confluence for 2–3 days in 10% serum medium before being submitted to the specific treatment (day 0) denoted by the lines

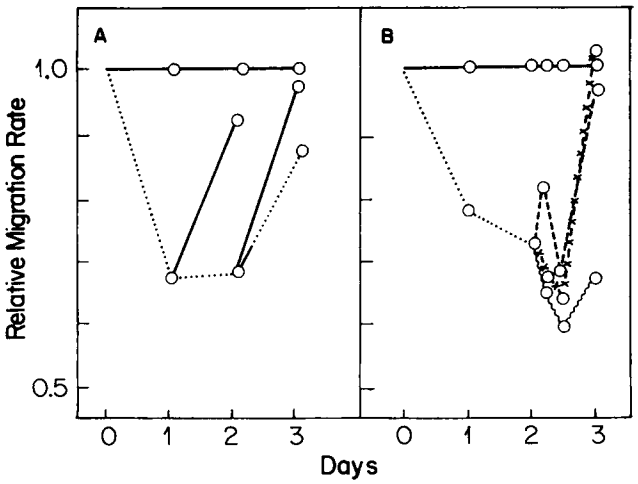

Fig. 2. Requirement of serum for the maintenance of nucleoid structure. [^{3}H]-thymidine-labeled confluent cells were supplied with medium containing 10% serum (——), 0.2% serum (·····), 10% serum plus 1 mM 3-aminobenzamide (----), 10% serum plus 1 mM cycloheximide, **B** (~~~), and 10% serum plus 20 uM ara-C, and 0.5 mM ddT (-x-x-). At the times indicated, the nucleoid migration rate was determined and normalized in relation to the control rate (○). Straight lines were arbitrarily drawn between the experimental points. Note that in one of the procedures in **A** the cells were exposed to 10% serum for only 4 h on day 2.

drawn with different symbols. For each experimental point, a control (maintained in 10% serum medium) was run simultaneously, and its migration rate was used as the reference value to normalize the results. Therefore, the horizontal solid line stands for fast nucleoids, which characterize confluent cells maintained in 10% serum medium. The actual migration rates of these controls did not vary by more than 10% in the different experiments, even when cells were resupplied with new medium. Figure 2A shows that upon maintenance in 0.2% serum medium for 1 or 2 days the resulting Go cells yielded slow nucleoids, whose migration rates dropped by about 35% in relation to the control. Fast nucleoids were again obtained upon reexposure of Go cells to 10% serum medium for 1 day. Even a short exposure of 4 h to this medium, after which the cells were resupplied with 0.2% serum medium, enabled the cells to yield fast nucleoids. Therefore, factors present in the serum have a crucial role in inducing chromatin to acquire a higher-order structure as revealed by the rapid sedimentation of nucleoids. Figure 2B shows that cycloheximide, a protein synthesis inhibitor, prevents chromatin structural changes in response to 10% serum medium. Inhibitors of DNA synthesis and DNA repair, ara-cyt plus ddT and 3-aminobenzamide, had no effect on the response to serum factors. One important observation was that plasma does not support the maintenance of higher-order chromatin structures, as shown in Figure 3. Equivalent results were obtained using three different plasma preparations (not shown). Plasma is essentially free of PDGF, and although it does maintain the cells in a viable state, it does not stimulate the replication of diploid fibroblasts [20]. That PDGF is important in maintaining a higher-order chromatin structure is demonstrated by the fact that PDGF-containing platelet extracts maintain nucleoids in a rapidly sedimenting state, in plasma medium, or in 0.2% serum medium (Fig. 3). Another competence factor, FGF, was also capable of causing this same tendency (Fig. 3).

It was recently reported that inhibition of protein synthesis forces early G1 cells to break down the program of cell proliferation by entering Go [26]. The experiment

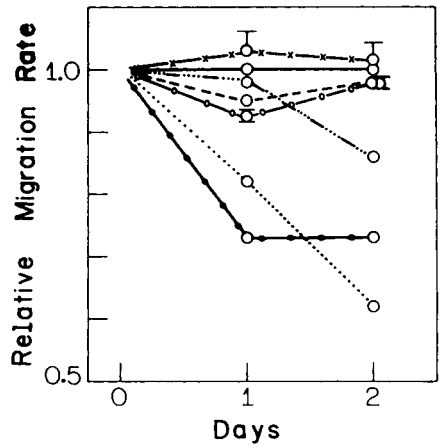

Fig. 3. PDGF and FGF support the maintenance of condensed chromatin domains. [^{3}H]-thymidine-labeled confluent cells were supplied with fresh medium containing 10% serum (——), 0.2% serum (·····), 5% plasma (—●—●), 0.2% serum plus 10 U ml^{-1} platelet extract (----), 5% plasma plus 10 U ml^{-1} platelet extract (—·—·—), 0.2% serum plus 2.4 U ml^{-1} FGF fraction (-x-x-), or 5% plasma plus 2.4 U ml^{-1} FGF (—O—O—). At the times indicated, the nucleoid migration rate was determined and normalized in relation to the control rate (O). Bars indicate deviation from the mean.

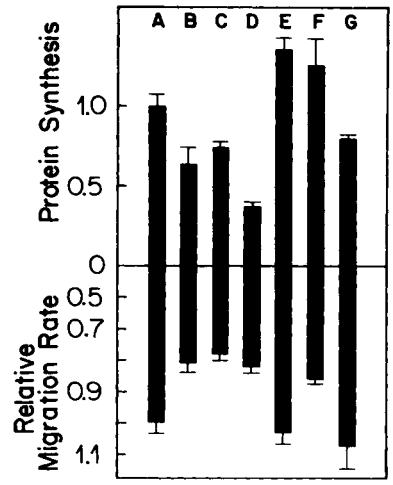

Fig. 4. Effects of growth factors on nucleoid condensation and protein synthesis. Parallel confluent cultures were used to determine nucleoid sedimentation rate and protein synthesis. After labeling with 0.2% uCi ml^{-1} [^{3}H]-thymidine, the cells were incubated in the indicated medium for 1 day, and the nucleoid migration rate was determined or the cells were incubated for 2 h with 5 uCi ml^{-1} [^{3}H]-leucine for the measurement of protein synthesis rate. **A,** 10% serum; **B,** 5% plasma; **C,** 0.2% serum; **D,** 10% serum plus 1 μM cycloheximide; **E,** 0.2% serum plus 2.4 U ml^{-1} of the fraction containing FGF; **F,** 0.2 serum plus 50% v/v v-sis conditioned medium (for protein synthesis) or 100% v-sis conditioned medium (for nucleoid migration rate); **G,** 5% plasma plus 100 ng ml^{-1} PMA. Bars indicate deviation from the mean.

illustrated in Figure 2B shows that protein synthesis is required for the induction of growth factor-mediated chromatin changes. However, normal levels of protein synthesis and condensed chromatin are not tightly linked events. Although this seems to be so under certain experimental conditions (Fig. 4), e.g., when cells are incubated in medium containing plasma (bar B), 0.2% serum (bar C), cycloheximide (bar D), or 0.2% serum plus FGF (bar E), the same is not observed with v-sis factor (bar F) or PMA (bar G). In the former case, the factor induced a normal level of protein synthesis, but this was insufficient to maintain higher-order chromatin structure. In the latter situation, PMA caused the nucleoids to sediment rapidly without inducing normal level of protein synthesis. PMA is a partial mitogen [6] and has been reported to activate protein kinase C, a step in PDGF signal transduction [27], and to induce expression of PDGF-inducible genes [28].

The authors attempted to determine whether or not these same transitions of chromatin structure occur in sparse cultures. When maintained in media deprived of growth factors, these cells break down the cycling program and enter Go. The proliferation rate can be estimated by the rate of [^{3}H]-thymidine incorporation into DNA. The results shown in Figure 5 show that a large decrease in [^{3}H]-thymidine incorporation occurs when sparse cells are cultured for 24 h in 0.2% serum (bar B), a symptom of cells having entered Go. As in the case of confluent cells, the corresponding nucleoids sediment slowly. If sparse cells are maintained in plasma for 24 h (bar C), proliferation is not so strongly inhibited, nor is the nucleoid migration rate extensively reduced. It is possible that in plasma part of the cells keep cycling and part are arrested at Go, giving rise to fast and slow nucleoids, respectively. In this case, the two different types of nucleoids would be expected to sediment according to a unimodal distribution, with an

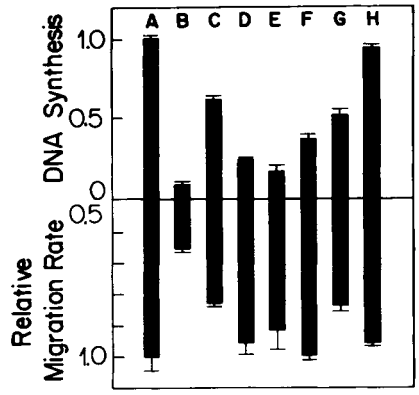

Fig. 5. Effects of growth factors on nucleoid supercoil domains and DNA synthesis. Cells were prelabeled for 24 h with 0.05 uCi ml^{-1} [^{14}C]-thymidine, replated at low density (18 x 10^3 cells cm^{-2}), and 18 h later supplied with the indicated media, where they remained for 24 h. After this period, the cells were pulse-labeled for 1 h in medium containing 10% serum and 5 uCi ml^{-1} [^{3}H]-thymidine and the rates of DNA synthesis ([^{3}H] cpm/[^{14}C] cpm of radioactivity incorporated into DNA) and nucleoid migration were determined. **A,** 10% serum; **B,** 0.2% serum; **C,** 5% plasma; **D,** 0.2% serum plus 2.4 U ml^{-1} of the fraction containing FGF; **E,** 0.2% serum plus 100 ng ml^{-1} PMA; **F,** 5% plasma plus 100 ng ml^{-1} PMA; **G,** 0.2% serum plus 50% v/v v-sis conditioned medium; **H,** 5% plasma + 50% v/v v-sis.

intermediate sedimentation rate. An important point in Figure 5 is that FGF counteracted the effect of low serum medium in maintaining higher-order chromatin structure (bar D), although it did not prevent the cells from becoming partially quiescent. PMA prevented the nucleoid change both in low serum and plasma (bars E and F). However, similar to FGF, PMA was not sufficient to permit the cells to cycle at a full rate. These results suggest that the chromatin response to growth factors is a direct response rather than a mere consequence of cell cycling. The presence of the polypeptide encoded by the v-sis gene effectively induced the nucleoids to become rapidly sedimenting either in low serum or in plasma (bars G and H). In the latter case the v-sis gene product was also capable of fully complementing the plasma in terms of stimulating cell proliferation. This result was expected, because v-sis is analogous to PDGF [22,23], the main competence growth factor missing in plasma.

An interesting question concerns the structural alterations of chromatin that lead to nucleoid unfolding in Go cells. A clear demonstration of the negative superhelicity of nuclear DNA can be given by titration with EtdBr. This drug intercalates into and untwists DNA, introducing positive supercoiling, which unwinds the loops; higher concentrations of EtdBr will overwind the DNA by supercoiling it in the positive direction [3,24]. Figure 6 shows that nucleoids of Go cells differ from nucleoids of cells maintained in normal medium in two respects: 1) in the absence of the intercalating agent Go nucleoids sediment slowly, as already shown; 2) as more EtdBr molecules are provided for intercalation, they unwind DNA loops. However, the titration point(full unwinding) and the appearance of right-handed superhelical DNA occurs at lower EtdBr concentrations in the case of Go cells nucleoids. For both nucleoids, high concentrations of EtdBr produced fast nucleoids with a high content of right-handed superhelical turns. These results indicate that relaxation of Go nucleoids is not due to breaks in DNA, in which case a lower content of right-handed superhelical DNA would

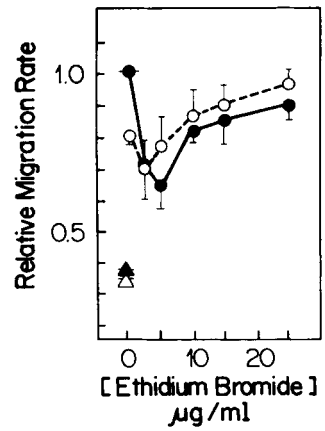

Fig. 6. Nucleoid sedimentation in gradients containing ethidium bromide. [^{3}H]-thymidine-labeled confluent cells were incubated for 2 days in medium containing 10% serum (●) or 0.2% serum (○). Nucleoids from 150,000 cells were sedimented in the presence of the indicated concentrations of EtdBr. In two parallel cultures, cells maintained in 10% serum (▲) or 0.2% serum (△) were treated with 4×10^{-4} M H_2O_2, which produces a frequency of DNA strand break [29] sufficient to relax all supercoil domains. Bars indicate deviation from the mean.

be induced by EtdBr. Rather, it seems that a lower content of left-handed superhelical turn is present in Go nucleoids, which are consequently titrated at lower concentrations of EtdBr in comparison with nucleoids of cells maintained in normal medium. For comparison, Figure 6 shows the sedimentation rates of both nucleoids when all supercoil domains were fully relaxed by DNA strand breaks produced by H_2O_2 [13,29]. Under these conditions, the difference in sedimentation rate was clearly eliminated. EtdBr titration curves of cycloheximide-treated cells yielded results very similar to those obtained with cells treated with low serum medium (results not shown).

DISCUSSION

The most important finding in the present experiments was that a higher-order chromatin structure is very sensitive to competence cell growth factors. A higher-order chromatin structure was found to be maintained in confluent or sparse cultures when any of the following factors were present: PDGF, FGF, v-sis gene product, and PMA. These factors share the ability to induce transcription of c-fos and c-myc genes [11,28,30], whose protein products are intracellular messengers of the competence growth factors [31]. Clearly, maintenance of the higher-order chromatin structure does not require progression of the cells along the cell cycle. In fact, only competence factors—not progression factors present in the plasma—are necessary and sufficient to establish this response. Moreover, in sparse cell cultures, FGF-induced chromatin folding in cells exposed to 0.2% serum medium, but it did not promote active proliferation.

The results establish a physiological distinction between arrested confluent cells and genuine Go cells. The former stop growing but continue to be sensitive to the competence factors present in the medium in that their chromatin sustains the higher-order structure; the latter occurring when the medium is deprived of competence factors and the chromatin structures become modified. This same condition can be attained by partial inhibition of protein synthesis with cycloheximide. It is interesting to note that the

cells did not need to be exposed to PDGF for a long time for the competent responses to be induced [32], nor did the folding of nucleoids require the permanent presence of PDGF (Fig. 2).

Other investigators have detected alterations in the DNA integrity of cells passing from a proliferation state to a quiescent or differentiated condition. DNA breaks appear during fibroblast fusion [14], starvation of Ehrlich tumor cells [33], and in quiescent lymphocytes [15,34]. Interestingly, the unfolding of supercoiled DNA when 3T3 fibroblasts enter Go is not caused by DNA strand breaks. In agreement with this, inhibitors of poly (ADP-ribose) synthesis and of DNA repair did not prevent the reestablishment of supercoiled DNA domains when 3T3 cells were stimulated to leave Go. It is possible that DNA strand breaks occur only in cells that are deeply quiescent or that are in the process of terminal differentiation. If not DNA breakage, three interpretations remain for the nucleoid change: 1) that rapidly sedimenting nucleoids induced by cell growth factors are due to specific proteins that attach to DNA and resist treatment with 2M NaCl and detergent; 2) that fast nucleoids result from general compaction changes; 3) that fast nucleoids result from DNA supercoiling in chromosomal loops. The present favor the latter interpretation because the difference in sedimentation rate between Go and serum stimulated cell nucleoids disappears when both are fully relaxed by DNA strand breaks produced by H_2O_2 (Fig. 6), a situation that would not be expected if attached proteins or general compaction changes were responsible for rapid sedimentation.

We do not know the mechanism involved in the folding/unfolding of supercoil domains of 3T3 cells. Two possibilities are: 1) that more superhelical turns accumulate in DNA than those restrained in the nucleosome, through the mediation of a topoisomerase; 2) if all superhelical turns are restrained by nucleosomes and other nucleosome-like structures [35], then the binding of extra proteins would allow extra torsional tension to be packaged in cycling cells and also through the mediation of a topoisomerase.

Warren and Cook [36] did not observe conspicuous variations in the level of superhelical turns in nucleoid DNA of cycling HeLa cells throughout the cell cycle. Similar results were obtained for 3T3 cells (unpublished results). It seems that the chromatin unfolding observed in the present investigation is a characteristic of Go state.

In the authors' opinion, analysis of nucleoid conformation provides a valuable tool in the study of signal transduction of growth factors, oncogene function, and control of cell proliferation. Elucidation of the molecular mechanisms of chromatin conformation changes may represent an important contribution to the understanding of the pleiotropic genetic control of cellular proliferation.

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