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Chromatin Structure and the Cell Cycle

(DNase I/synchronized HeLa cells/monkey-kidney cells/contact inhibition)

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ABSTRACT **Pancreatic DNase I is used to probe the structure of chromatin isolated from synchronized HeLa cells. The degree to which DNA in chromatin is protected from DNase attack varies during the G_1, S, and G_2 phases of the cell cycle. In addition, the DNase sensitivity of chromatin from contact-inhibited African green monkey kidney cells differs from that of actively dividing, subconfluent cultures. These cell cycle-dependent chromatin changes were observed consistently at all enzyme concentrations (5000-fold range) and incubation times (15 min–2 hr) tested. The results indicate that the degree of complexing between DNA and chromosomal proteins changes during interphase, and they suggest that the chromosome coiling cycle of visible mitosis may extend in more subtle form over the entire cell cycle.**

Recent studies of [^{3}H]actinomycin binding in synchronized HeLa cells have resolved subtle changes in chromatin structure during interphase (G_1, S, and G_2 phases), when there is no cytological evidence of chromosome coiling (refs. 1 and 2 and Fig. 1). Binding per unit of DNA increases gradually throughout the G_1 phase, reaches a maximum in early S, and then declines sharply throughout the remainder of S, attaining a minimum value in G_2 and mitosis. Since the illustrated pattern was observed in assays on living cells, on ethanol-fixed cells, and on isolated nuclei incubated with [^{3}H]actinomcyin *in vitro*, but not with purified DNA, it implied changes in the degree of complexing between DNA and chromosomal proteins during the cell cycle. This possibility has been explored by the use of DNase.

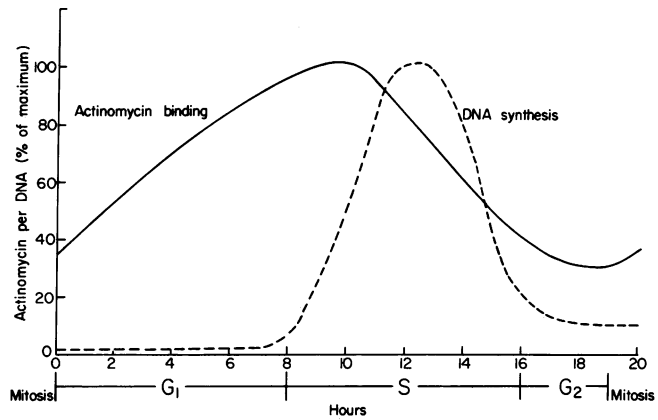

FIG. 1. Actinomycin binding in synchronized HeLa cells. Redrawn from curves and data in refs. 1 and 2; the cyclic pattern shown was obtained regardless of whether the [^{3}H]actinomycin binding was measured in living cells, ethanol-fixed cells, or isolated nuclei *in vitro*. [^{3}H]Actinomycin binding and DNA synthesis, both as % of maximum.

METHODS

Cells and Synchronization. HeLa cells (S_3 strain) were maintained in suspension culture at 2 to 4 $\times$ 10^5 cells/ml by daily dilution with fresh medium (3) containing 3.5% each of calf and fetal-calf serum. Synchronization was by the double thymidine technique (4), as detailed elsewhere (5). BSCb cells, a heteroploid, but contact-inhibited, line derived from African green monkey kidney (6), were propagated in Blake bottles with Dulbecco's modified Eagle's minimal essential medium containing 10% calf serum; cultures were fed every 2 days regardless of cell density. BSCb cells were harvested by rinsing the cell sheet with calcium- and magnesium-free medium without serum, followed by trypsinization.

Cell Fractionation and Chromatin Isolation. Cells were collected by low-speed centrifugation, washed twice in cold Earle's balanced salt solution (7), and disrupted by Dounce homogenization in 0.01 M NaCl–1.5 mM $MgCl_2$–0.01 M Tris·HCl, pH 7.0. Nuclei were pelleted at 1000 $\times$ g (3 min), and washed three times in the same buffer. A detailed description of the method for isolation of chromatin will be published elsewhere (Bhorjee and Pederson, in preparation). Briefly, HeLa or BSCb nuclei in buffer are disrupted by sonication and centrifuged in 30% sucrose at 4500 $\times$ g for 15 min, which selectively pellets nucleoli. The material remaining on top of the sucrose, which contains ribosomal and heterogeneous nuclear ribonucleoprotein particles, nuclear membrane fragments, and chromatin, is removed and centrifuged through 60% sucrose at 135,000 $\times$ g for 90 min. The pellet contains 82–90% of the nuclear DNA, trace amounts of RNA, and has a protein to DNA ratio of 1.3–1.6; contamination by cytoplasmic proteins is less than 2.0% by mass. Ribosomal structural proteins and heterogeneous nuclear RNA-associated proteins, which are easily demonstrable by electrophoresis of the nuclear sonicate, are absent from the final chromatin preparation.

Purification of HeLa DNA. DNA was isolated from HeLa nuclei by the "sodium dodecyl sulfate–Pronase" technique (8), followed by chloroform deproteinization. Final traces of protein were removed by banding the DNA in CsCl (initial ρ = 1.742 g/cm^3; 368,000 $\times$ g, 36 hr); fractions containing the A_{260} peak were pooled and dialyzed overnight against buffer to remove CsCl.

DNase Digestions. Chromatin samples were dialyzed overnight against buffer and adjusted to contain 15–50 μg of

DNA/ml (as A_{260}); in a given set of assays all of the chromatin samples contained equal amounts of DNA per ml. To 1.0 ml of chromatin, or pure DNA, was added 0.10 ml of buffer containing 11 times the desired final concentration of DNase; the tubes were stoppered and incubated at 37° for 15 min–4 hr. Most experiments were performed with pancreatic deoxyribonuclease I (electrophoretically-purified, RNase-free); some were done with staphylococcal nuclease (*Staphylococcus aureus*), both from Worthington Biochemical Corp. (Freehold, N.J.). At the desired time, digestions were stopped by the addition of cold perchloric acid to 0.25 M, and the samples were centrifuged at 37,000 × *g* for 15 min to deposit the acid-precipitated, undigested chromatin. The supernatants were then aspirated off and the amount of DNA hydrolyzed was determined by measurement of the absorbance at 260 nm or, in those cases where the cells had been labeled to equilibrium with [^{3}H]thymidine, the amount of radioactivity in the acid-soluble fraction was determined by liquid scintillation counting.

RESULTS

DNase digestion of chromatin from synchronized cells

Fig. 2 compares the digestion of HeLa DNA and chromatin after incubation for 2 hr with different concentrations of pancreatic DNase. Clearly, DNA in chromatin is afforded a significant degree of protection from DNase attack. However, the degree of this protection need not be the same for all chromatin preparations; for example, Mirsky found differences in the DNase sensitivity of calf-thymus chromatin isolated by two methods (9). The present experiments ask instead whether chromatin samples prepared at different stages

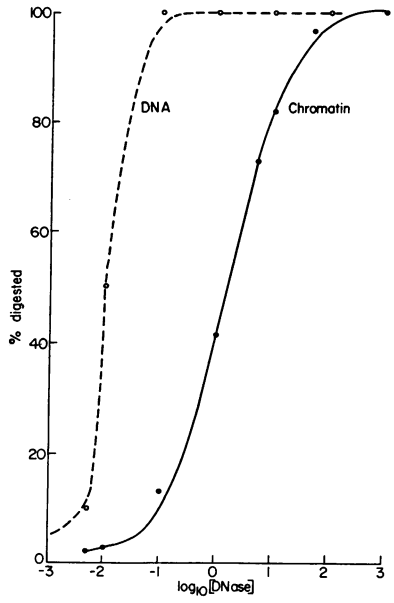

FIG. 2. Digestion of HeLa chromatin and DNA by pancreatic DNase I. HeLa cells were labeled for two generations with [^{3}H]-thymidine (0.05 μCi/ml). 1.0-ml samples of DNA or chromatin were incubated for 2 hr at 37.5° with various concentrations of pancreatic DNase I. After perchloric acid was added to 0.25 M the samples were centrifuged (37,000) × *g*, 15 min), and the radioactivity in the supernatant was determined by liquid scintillation counting. The percentage of DNA digested was calculated from the radioactivity of the input DNA or chromatin. O——O, DNA; ●——●, chromatin.

of the cell cycle have similar responses to DNase, or rather display digestion profiles that are transposed along the abscissa. The results shown in Fig. 3 reveal that the latter is the case. The digestion profile of chromatin isolated from cells in early S (2 hr in *inset*) lies significantly to the left of that for late S (8 hr) or early G_1 (17 hr) phases. The profiles differ by about 0.3 $\log_{10}$ unit, indicating that twice the DNase concentration was required to obtain the same amount of digestion in the late S and early G_1 samples as in early S samples. To be certain that the transposition of the early S curve to the left was not a peculiarity of recovery from thymidine blockade, a separate experiment was performed in which chromatin was prepared from cells entering S phase in the second cycle (24 hr in *inset*) and was compared to G_2 and early G_1 samples. The results were identical to those depicted in Fig. 3. The relative DNase sensitivities of chromatin from synchronized cells were also measured as a function of time, with enzyme concentration held constant. The rates of digestion (Fig. 4) illustrate the same trends as were evident in Fig. 3: early S chromatin is digested faster than that from mid-S or G_2 phases. As expected, chromatin from nonsynchronized cells ("random") displays an intermediate response.

It appears that there are two steps in the digestion rate, with 30–40% of the DNA being hydrolyzed in the first 15 min. However, the amount of DNA digested in 15 min is itself a function of DNase concentration (Fig. 5 and ref. 9), and there

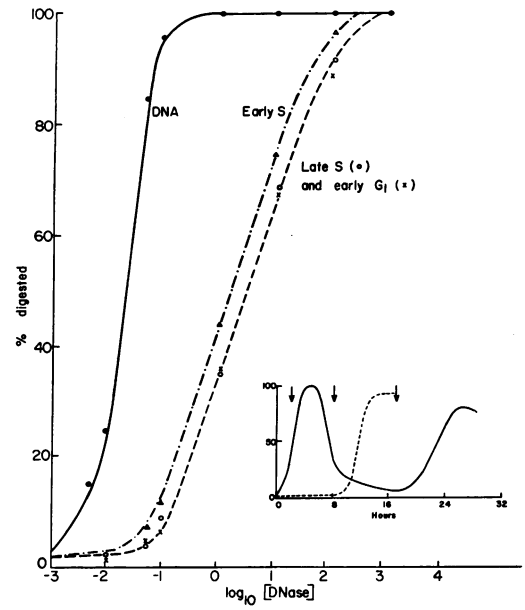

FIG. 3. DNase digestion profiles of chromatin from synchronized cells. 2000 ml of HeLa cells at 3 × 10^5/ml were synchronized by the double-thymidine procedure; after 16 hr in 2 mM thymidine, the cells were resuspended in fresh medium at 3 × 10^5/ml and labeled for 10 hr with [^{3}H]thymidine (0.5 μCi/ml). Cold thymidine was again added to 2 mM, and 12 hr later the block was released by resuspension in fresh medium (0 hr, *inset*). Synchrony was monitored by measurement of DNA synthesis and mitosis as described (2). At the times indicated (*arrows*), one-third of the cells were harvested and chromatin was isolated. DNase digestion assays were performed in duplicate as described in *Methods;* each point is the average of the duplicates. The late S and early G_1 points were so similar that for clarity a single curve is drawn. *Inset;* ——, % DNA synthesis; – – –, % metaphase arrest.

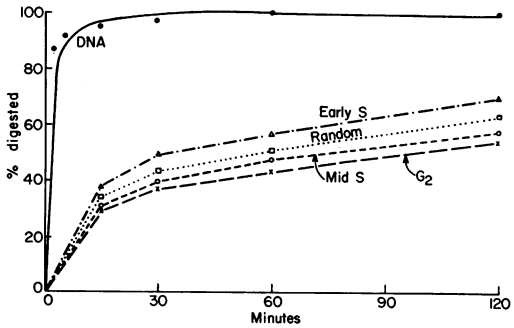

FIG. 4. Rate of digestion of chromatin from synchronized cells. A culture was synchronized and labeled as in Fig 3; portions were harvested at 2 hr after release of the second thymidine block (early S), 4.5 hr (mid-S), and 9 hr (G_2), when DNA synthesis was 15% of the peak mid-S value and only 12% of the cells were in metaphase-arrest (colchicine). Chromatin was also prepared from random cells labeled for 24 hr with [^{3}H]thymidine. Assays were performed with DNase at 5 μg/ml.

is no indication of two-step kinetics with a second enzyme, staphylococcal nuclease (Fig. 5). These considerations point up the importance of making measurements over the full spectrum of DNase action. It is clear that the cell cycle-changes in DNase sensitivity are as consistent as a function of time (Fig. 4) as they are with regard to enzyme concentration over a 5000-fold range (Fig. 3). In both cases, a time-concentration continuum of DNase action has been used to reveal *relative* differences in chromatin structure.

DNase sensitivity of newly-replicated DNA

The data in Figs. 2–5 are based upon the release of acid-soluble radioactivity from chromatin after it was labeled *in vivo* with [^{3}H]thymidine for 1.5 cell generations. In other assays (not shown), digestion was monitored by release of A_{260}, to yield results identical to those in Figs. 3 and 4. These procedures thus measure the average response of all DNA in each chromatin sample. It is important to determine the degree of variation that yields this average; DNase sensitivity was, therefore, measured in a situation where the digestion of a specific class of DNA could be visualized separately from that of the bulk DNA. Randomly growing cells were pulse-labeled for 15 min with [^{3}H]thymidine, and chromatin was isolated.

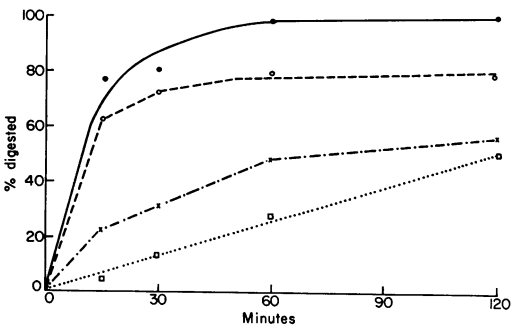

FIG. 5. Kinetics of DNase action on HeLa chromatin. [^{3}H]-Thymidine-labeled chromatin from random HeLa cells was incubated with pancreatic DNase I at 0.5 (X– – –X), 10 (O– – –O), or 25 μg/ml (●——●) or with staphylococcal nuclease at 5 μg/ml (□– – –□) for various times. Percentage of DNA digested was calculated from input radioactivity.

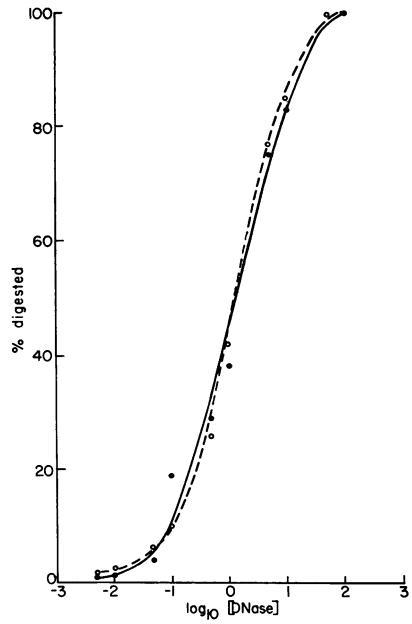

FIG. 6. DNase sensitivity of chromatin containing newly-replicated DNA. 2500 ml of random HeLa cells were concentrated 10-fold and pulse-labeled for 15 min with [^{3}H]thymidine, 2 μCi/ml; care was taken to maintain the pH at 7.2. After incubation of chromatin, the samples were centrifuged at 37,000 × *g* for 30 min; the supernatants were aspirated, their absorbance at 260 nm was determined, and the amount of radioactivity was then measured by liquid scintillation counting. ●——●, ^{3}H; O– – –O, A_{260}.

The digestion profile of newly-replicated DNA (^{3}H) was identical to that of the total DNA (A_{260}) (Fig. 6). While it is possible that some replicating DNA was lost during chromatin isolation, perhaps as a result of real or adventitious binding to other nuclear constituents, the results in Fig. 6 show that the DNase digestion profile of total chromatin would not be transposed on the abscissa by the presence of newly-replicated DNA in the chromatin samples. The sensitivity of HeLa chromatin to DNase is thus linked in some way to the cell cycle (Figs. 3 and 4), but apparently not through DNA replication *per se*.

Growing versus contact-inhibited monkey-kidney cells

Various cytochemical measurements have indicated that chromatin in nondividing cells is less accessible to dyes and other ligands than in growing cells (10–13). Therefore, this point was explored with DNase. Chromatin was purified from sparse and confluent cultures of BSCb cells; the measured rate of DNA synthesis per cell in the confluent cultures was 5% of that in the low-density cultures (both sets of cultures were maintained on identical feeding schedules to minimize nutritional and pH variations). The data in Fig. 7 and Table 1 demonstrate that the DNase digestion profile of chromatin from growing cells lies significantly to the left (more sensitive) of that from contact-inhibited cells. The effect was consistent at all enzyme concentrations tested, and there was good agreement between the two experiments. Since the profile for growing cells represents the average of all cell cycle stages, the rightward transposition of the profile for confluent chromatin suggests that the majority of cells were arrested at a cell cycle

position of minimal DNase sensitivity, probably early G_1 (14, 15).

DISCUSSION

The present experiments demonstrate changes in the DNase sensitivity of chromatin isolated at different stages of the cell cycle, or from growing or "contact-inhibited" populations. It is relevant to discuss these findings in relation to recent results of others concerning the use of DNase as a probe for studying chromatin structure. Clark and Felsenfeld reported that 50% of the DNA in calf-thymus chromatin was attacked by pancreatic DNase or staphylococcal nuclease, and that a similar fraction was available to complex with poly(D-lysine) (16). On the other hand, Mirsky found that the amount of DNA digested was entirely dependent upon DNase concentration and time of incubation, so that conclusions concerning the absolute amount of unprotected DNA in chromatin were unwarranted (9). My results with HeLa chromatin are essentially similar: DNA in chromatin responds to DNase attack continuously with respect to enzyme concentration and time of incubation. We should recall that various studies with DNA ligands have demonstrated that there are 2- to 3-times more available binding sites in pure DNA than in chromatin (17–22); thus, the conclusion that a significant fraction of the DNA in chromatin is *biochemically* unreactive does not rest entirely upon the use of DNase. In a recent study, Schmidt *et al.* (23) found that calf-thymus chromatin was digested by DNase in two steps, a fast initial phase and a slower second component, as is suggested for HeLa chromatin in Fig. 4. As these authors used but one DNase concentration (10 μg/ml), their two-step kinetics at that concentration cannot be used to draw conclusions on the absolute amount of "free" DNA, due to the criticism of Mirsky (ref. 9, and see Fig. 5). However, Schmidt *et al.* also demonstrated that at the end of the first phase of digestion, when about 50% of the DNA had been hydrolyzed, the remaining chromatin had a ratio of anionic to cationic groups of 1.0, while the initial chromatin had a ratio of 2.0. The implication was that an anionic chromatin constituent (DNA) had been digested without loss of cationic groups (histones). Thus, the possibility certainly exists that although the action of pancreatic DNase on chromatin is a continuum, at certain times or concentrations of enzyme one type of DNA is digested preferentially. In addition, it seems probable that the degree of dispersion of the chromatin at the time of DNase assay is a key factor; a particular complication would be the formation of networks of chromatin threads in highly concentrated samples. In these cases, DNase could discriminate between single fibers, which would be more sensitive to attack, and bundles of fibers, which would be more resistant, rather than between different regions along the longitudinal axis of individual fibers. Clearly, further work will be required to clarify these points. In the present study, DNase was not used to measure the fraction of DNA *ultimately* digestible, for this was always 100%, but instead to monitor relative rates of digestion as a function of either time or DNase concentration. Used in this fashion, DNase has resolved changes in the structure of chromatin during the cell division cycle.

TABLE 1. *Digestion of chromatin from growing and confluent monkey-kidney cells*

DNase (μg/ml)	% DNA digested					
	0.01	0.10	1.0	5.0	10.0	100.0
Growing	12	26	57	—	92	100
Confluent	8	20	47	69	73	100
Growing	11	36	61	69	77	100
Confluent	9	29	47	61	68	98

For each experiment four confluent cultures (about 2×10^7 cells/Blake bottle) and 16 sparse cultures (about 4×10^6/bottle) were harvested by mild trypsinization, and chromatin was prepared as detailed in *Methods*. Both sets of cultures had been planted 4 days previously from the same parent stock and refed on day 2, so that the main variable in the comparison was cell density. DNase digestion assays were performed as described in Fig. 2, except that because of the limited amount of chromatin available and the necessity of using A_{260} to monitor the DNase digestion, a single assay was performed at each enzyme concentration in each of the two experiments. DNA synthesis was measured in growing and confluent cultures by addition of [^{3}H]-thymidine to 0.5 μCi/ml for 30 min, rinsing the cells with cold calcium- and magnesium-free "DME salts" (Dulbecco-modified Eagle's medium minus amino acids and vitamins), and harvested by trypsinization. After the cells were counted, aliquots were removed and mixed with cold 10% trichloroacetic acid; the precipitates were collected on nitrocellulose filters, which were than dissolved in 10 ml of Bray's fluid for liquid scintillation counting. Incorporation was expressed as cpm/10^6 cells. By this procedure, the rate of DNA synthesis per cell in the confluent cultures was 5% of that in growing cultures. While no corrections were made for possible differences in thymidine uptake or intracellular TMP pools, these data are in agreement with the respective mitotic indices as determined by phase-contrast microscopy.

The present experiments do not attempt to elucidate the specific macromolecular changes in chromatin that confer cell cycle-dependent alterations in DNase sensitivity. Post-translational modifications of histones or nonhistone chromosomal

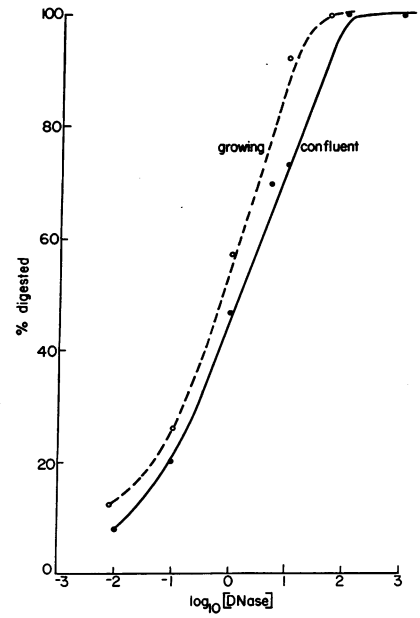

FIG. 7. DNase sensitivity of chromatin from growing and nongrowing monkey-kidney cells. Digestion was monitored by measurement of the release of acid-soluble A_{260} as in Fig. 6. The data plotted are those of the first experiment in Table 1, where the experimental details are given.

proteins (24–26) could play important roles. It should be noted, however, that the factors that restrict DNase action on chromatin need not necessarily be proteins, or even macromolecules, although these are reasonable candidates. For example, *in vivo* differences in chromatin-bound metal ions (27) could persist throughout the isolation procedure and confer different degrees of stabilization on the chromatin at the time of DNase assay.

It is to be noted that the sensitivity of chromatin to DNase, as measured in this investigation, reflects the response of total chromatin. While estimates of the amount of DNA that is transcribed in various eukaryotic cells are only approximate (28), it seems unlikely that changes in transcriptional activity during the cell cycle would be reflected by structural alterations in all of the chromatin. Furthermore, the experiment shown in Fig. 6 indicated that the changes in DNase sensitivity are not due to the presence of newly-replicated DNA in the chromatin samples. I would suggest instead that the interphase patterns of actinomycin binding (1, 2) and DNase sensitivity are manifestations of a continuous "chromosome cycle," which can be visualized by microscopy only during mitosis (prophase–telophase), but which is resolved by more sensitive probes during interphase as well (G_1, S, G_2). The concept of such a cycle is not new (29), and is compatible with other data, particularly on the responses of synchronized mammalian cells to x-irradiation (30). The results on chromatin from contact-inhibited cells suggest that this chromosome cycle can be interrupted in nondividing, "G_0" cells, perhaps under the control of the cell surface; the reinitiation of the "chromosome cycle" in such cells may then lead to those events that we know as the cell cycle (DNA replication and mitosis). Clearly, an actual causal relationship between a cycle of structural transitions in interphase chromosomes and the overall process of cell replication remains to be determined.

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