

# CHROMATIN STRUCTURE AND THE CELL DIVISION CYCLE

## Actinomycin Binding in Synchronized HeLa Cells

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### ABSTRACT

Measurements of actinomycin-<sup>3</sup>H binding in synchronized HeLa cells reveal that the binding capacity of chromatin decreases progressively during the S phase despite a doubling of nuclear DNA content, reaches a minimal level during G<sub>2</sub> and mitosis, and then increases gradually throughout the subsequent G<sub>1</sub> interval. Since this pattern was evident in experiments with living cells, ethanol-fixed cells, and isolated nuclei, but not with purified DNA, the actinomycin binding profile may reflect changes in the degree of association between DNA and chromosomal proteins at different stages of the cell cycle.

### INTRODUCTION

A number of studies have indicated that the binding of actinomycin to DNA of living or fixed cells is significantly influenced by the intranuclear organization of the chromatin (Darzynkiewicz, Bolund, and Ringertz, 1969; Brachet and Hulin, 1969; Berlowitz, et al., 1969; Bolund, 1970); in general, highly condensed chromatin binds considerably less actinomycin than the same amount of DNA disposed in a more diffuse configuration. We have employed actinomycin-<sup>3</sup>H to study the organization of chromatin over the cell division cycle in synchronized HeLa cells and have found significant changes in binding during *interphase*, when there is no cytological evidence of chromosome condensation. Our results suggest that in this case actinomycin-<sup>3</sup>H binding may be a measure of the degree of complexing between DNA and chromosomal proteins, and that this parameter changes during the cell cycle.

### MATERIALS AND METHODS

#### *Cells and Synchronization*

HeLa cells (S<sub>3</sub> strain) were grown in suspension culture using Eagle's medium (Eagle, 1959) sup-

plemented with 3.5% each of calf and fetal calf serum. Populations synchronized in S and G<sub>2</sub> were obtained by double thymidine blockade (Puck, 1964), and those in mitosis and G<sub>1</sub> were prepared by selective detachment of metaphase cells from partially confluent monolayer cultures (Robbins and Scharff, 1966; Pederson and Robbins, 1971).

#### *Measurement of Actinomycin Binding in Cells and Nuclei*

Assays of actinomycin binding were carried out in both living and ethanol-fixed cells. In the former case, 25 ml samples containing approximately  $4 \times 10^8$  cells/ml were incubated at 37°C with actinomycin-<sup>3</sup>H at concentrations ranging from 1 to 40  $\mu$ Ci/ml (total actinomycin concentration 0.3–12  $\mu$ g/ml) for 15 min–4 hr and then washed twice in cold Earle's balanced salt solution (Earle, 1943). To insure complete removal of free actinomycin D (AMD)<sup>1</sup>, the cell pellet was extracted three times in cold 95% ethanol for 15 min each; these conditions were chosen

<sup>1</sup> The following abbreviations are used in this paper: AMD, actinomycin D; BUdR, 5-bromodeoxyuridine; RSB, reticulocyte standard buffer (0.01 M NaCl, 0.0015 M MgCl<sub>2</sub>, 0.01 M Tris-HCl, pH 7.2); SDS, sodium dodecyl sulfate.

because AMD is more soluble in ethanol than water and has a negative temperature coefficient of solubility (Gellert et al., 1965). Negligible amounts of AMD-<sup>3</sup>H were removed in the third extraction; when these extracted cells were incubated overnight in cold ethanol, approximately 90% of the AMD-<sup>3</sup>H remained insoluble. The specificity of the ethanol-insoluble binding was determined by resuspending the extracted cells in reticulocyte standard buffer (0.01 M NaCl, 0.0015 M MgCl<sub>2</sub>, 0.01 M Tris-HCl, pH 7.2) (RSB) containing 100 μg/ml DNase I; this and a replicate sample without enzyme were incubated at 37°C for 1 hr, reextracted twice in cold ethanol, and the AMD remaining was measured by liquid scintillation counting. By this procedure it was determined that approximately 94% of the ethanol-insoluble actinomycin was bound to DNase-sensitive macromolecules. The total uptake of AMD-<sup>3</sup>H (free plus bound) by living cells was measured by centrifuging the suspension, without washing, in a specially-designed hematocrit tube and counting samples of both the cell pellet (resuspended in 1.0 ml of Earle's solution) and the supernatant medium. Radioactivity in the pellet was corrected for an extracellular space of 17%, as determined with inulin-<sup>3</sup>H, and expressed as μmoles AMD/mg packed wet cells. We emphasize the importance of the ethanol extractions in the AMD binding assays since less than 15% of the total AMD taken up by the cells is removed during three washes in Earle's solution; only after removing this free AMD with ethanol can the cell cycle-dependent changes in ligand binding be visualized.

In some cases it was desirable to eliminate the possibility of enzymatic modifications of AMD in living cells by fixing them in cold 70% ethanol for 30 min before the binding assays; the fixed cells were then resuspended in 95% ethanol, incubated with actinomycin-<sup>3</sup>H (5–20 μCi/ml) for 1 hr at 4°C, and extracted with cold ethanol as above. To study AMD binding in isolated nuclei, synchronized cells were washed in Earle's solution and disrupted by Dounce homogenization in RSB; the nuclei were deposited at 1000 g for 3 min, washed twice in RSB, and resuspended at  $2.5 \times 10^7$  nuclei/ml. After incubation with AMD-<sup>3</sup>H (20 μCi/ml, 1 hr at 4°C), the nuclei were washed once in RSB and extracted in cold 95% ethanol as above.

#### *Binding of Actinomycin-<sup>3</sup>H to Purified DNA*

Nuclei were isolated from synchronized cells as above and DNA purified by the "SDS-pronase" method (Maio and Schildkraut, 1967) using one phenol extraction (20°C) followed by three with chloroform; the last aqueous phase was made 67% in ethanol, stored at least 2 hr at -20°C, and the precipitated DNA was collected by centrifugation at 12,000 g for 15 min. The DNA was dissolved in RSB

and dialyzed for 16 hr at 4°C against RSB to remove traces of chloroform and ethanol; the samples were then adjusted to contain equal amounts of DNA as measured by diphenylamine. Actinomycin binding assays were carried out by adding 0.04 ml of actinomycin-<sup>3</sup>H (20 μCi or approximately 4.0 μg AMD) to 0.96 ml of RSB containing 1 μg of DNA; under these conditions the total AMD concentration corresponds to about one molecule per DNA nucleotide. After 5–30 min at 4°C, the samples were mixed with 5 ml of cold 95% ethanol and poured through a nitrocellulose filter (0.45 μ pore size), which was then washed five times with 5 ml of ethanol. To determine whether traces of free actinomycin were still retained by the filter, incubations were performed as above but without DNA; the amount of radioactivity on these filters after three ethanol washes was then subtracted from the values for the DNA-containing samples. We again wish to emphasize the importance of accounting for, and removing if possible, free actinomycin. For example, when incubation mixtures formulated as above were poured directly onto presoaked filters, large amounts of free actinomycin were retained in an ethanol-insoluble form; similarly, when samples of DNA were attached to filters and then incubated in actinomycin, very high blanks were recorded. Only the procedure of making the incubation mixture 80% in ethanol *before* filtration resulted in blank values low enough for the DNA-bound actinomycin to be measured precisely.

#### *Isopycnic Banding of DNA-AMD Complexes in CsCl Gradients*

Isolated nuclei were incubated with AMD-<sup>3</sup>H as detailed above, washed three times in cold RSB, and disrupted by sonication (30 sec at 40 watts, Heat Systems-Ultrasonics, Inc., Plainview, N. Y., model W185). A portion of the sonicate containing approximately 50 μg of DNA, measured by diphenylamine, was adjusted to a volume of 8.0 ml with RSB and mixed with 10.0 g of CsCl, for a final density of 1.742 g/cc; the samples were then centrifuged in a Beckman Spinco fixed angle 65 rotor as detailed in the figure legend. Gradients were collected into 0.25 ml fractions while continuously monitoring the absorbance at 260 mμ with a Gilford recording spectrophotometer. The fractions were adjusted to 1.0 ml and dialyzed against water for 4 hr to remove CsCl; radioactivity was measured by liquid scintillation counting with Bray's fluid.

#### *Isotopes and Chemicals*

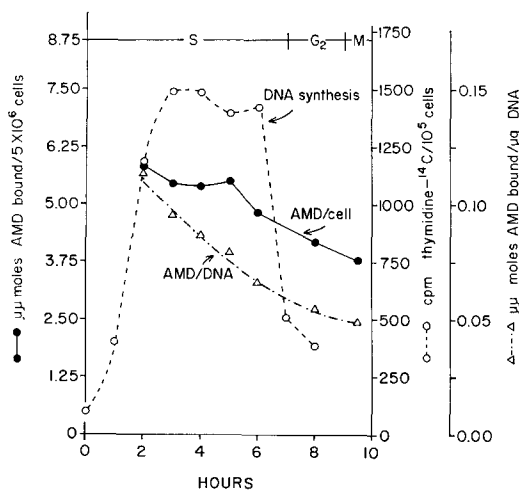
Actinomycin-<sup>3</sup>H (8.4 Ci/mmole), thymidine-<sup>14</sup>C (methyl labeled, 40 mCi/mmole), BUdR (5-bromodeoxyuridine), and optical grade cesium chloride were obtained from Schwarz/Mann, Orangeburg,

N. Y. Pancreatic deoxyribonuclease (DNase I), pancreatic ribonuclease, and ribonuclease T<sub>1</sub> (*Aspergillus oryzae*) were purchased from Worthington Biochemical Corp., Freehold, N. J.

## RESULTS

### Binding of AMD-<sup>3</sup>H in Synchronized Cells and Nuclei

Fig. 1 illustrates the results obtained when synchronized cells were exposed to AMD-<sup>3</sup>H during the S, G<sub>2</sub>, and mitotic phases of the cell division cycle. Binding per cell decreased by about 15% between the beginning and end of S, despite



**FIGURE 1** Actinomycin binding during S, G<sub>2</sub>, and mitosis. Cells growing at  $2-4 \times 10^5$ /ml were synchronized by the double thymidine procedure (14 hr-10 hr release-12 hr). At hourly intervals two samples of cells were concentrated to  $10^7/2.5$  ml and each incubated at 37°C with  $20 \mu\text{Ci/ml}$  actinomycin-<sup>3</sup>H for 1 hr. DNA synthesis was monitored by pulse labeling separate samples ( $4 \times 10^5$  cells) for 15 min with  $1.0 \mu\text{Ci}$  of thymidine-<sup>14</sup>C and measuring the amount of radioactivity incorporated into cold 10% trichloroacetic acid-precipitable material. Colchicine,  $0.2 \mu\text{g/ml}$ , was added to the population at 6 hr to accumulate subsequently-dividing cells in metaphase (control experiments revealed no effect of the drug on actinomycin binding in either interphase or mitotic cells). Also assayed at hourly intervals, but not plotted, was total actinomycin uptake as detailed in the text; these measurements revealed that the observed binding pattern is not due to differences in the cells' permeability to actinomycin. ●—●, actinomycin binding per cell, (average of two replicates); △---△ binding per unit of DNA; ○---○, thymidine-<sup>14</sup>C incorporation.

a near doubling of DNA/cell during this interval as determined by diphenylamine assays on separate samples. When DNA replication is taken into account, the amount of actinomycin/DNA is seen to decrease by approximately 50% throughout S and by another 20% by the time the population has reached mitosis; the mitotic sample in Fig. 1 was assayed 3 hr after the end of S, at which time 92% of the cells were observed to be in metaphase-arrest by phase contrast microscopy. The unlikely possibility that these results were generated by a metabolic transformation of the actinomycin to a nonbinding form as the population progressed through S was ruled out by using ethanol-fixed cells for the binding assays, in which case the results were the same as depicted in Fig. 1. Furthermore, the binding pattern cannot be explained by a gradually increasing contamination of the S population with mitotic cells, since the mitotic index remained below 5% until the end of G<sub>2</sub> (about 9 hr) in all experiments. It is thus clear that as cells progress through the S and G<sub>2</sub> phases of the cell cycle, the number of available AMD binding sites in the chromatin decreases; this trend precedes by many hours the time of chromosome coiling (prophase), when an abrupt decrease in binding might have been expected.

That actinomycin binding reflects a more subtle aspect of chromosome architecture than the degree of gross condensation is also supported by data for cells in mitosis and G<sub>1</sub> (Fig. 2). AMD binding per cell increases gradually throughout G<sub>1</sub> and reaches a maximum value at the second to third hour of S; a shoulder in the G<sub>1</sub> binding profile was consistently noted at 4-6 hr after mitosis. Since microscopy reveals that the mitotic (condensed) chromosomes had reverted to the uncoiled, interphase state within 30 min after telophase, the binding increase during G<sub>1</sub> cannot be attributed to any grossly visible modification of chromatin.

The pattern of AMD binding illustrated in Fig. 1 was also observed when the assays were performed with isolated nuclei (Table I). Equal numbers of nuclei from early and late S phase cells bound approximately equal amounts of AMD-<sup>3</sup>H although the late S sample contained almost twice as much DNA. After isolation in the hypotonic buffer RSB, HeLa nuclei contain chromatin which is homogeneously diffuse (Holtzman et al., 1966); thus the difference in AMD binding/DNA illustrated in Table I cannot be attributed to local

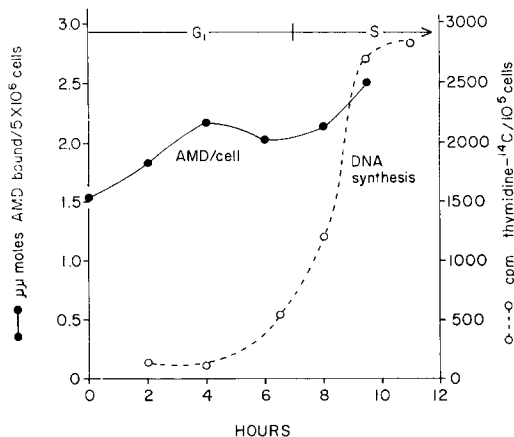


FIGURE 2 Actinomycin binding during the mitotic-G<sub>1</sub> region of the cell cycle. At 0 hr approximately  $7 \times 10^7$  mitotic cells were obtained by selective detachment from partially confluent monolayers. At each time point  $1.2 \times 10^7$  cells ( $6 \times 10^6$  for the 0 hr sample since cytokinesis had not yet occurred) were fixed in 70% ethanol as detailed in text, divided into two equal portions, and then incubated for 1 hr with actinomycin-<sup>3</sup>H (20  $\mu$ Ci/ml) at 4°C. Each point represents the average value of the replicate samples. DNA synthesis was monitored as detailed in Fig. 1. ●—●, actinomycin bound per cell; ○---○, thymidine-<sup>14</sup>C incorporation.

changes in the intranuclear organization of chromatin between early and late S (e.g., heterochromatinization).

#### Analysis of DNA-Actinomycin Complexes in CsCl Gradients

To study this phenomenon further, the DNA-AMD complexes formed in cells or nuclei were analyzed by isopycnic banding in CsCl gradients. When thymidine-<sup>14</sup>C-labeled nuclei from random cells were incubated with AMD-<sup>3</sup>H, disrupted by sonication and centrifuged in CsCl, 35–60% of the actinomycin initially bound remained attached to the banded DNA. This variable recovery precluded a direct comparison of samples from different points in the cell cycle, since the actual changes in the AMD/DNA ratio would not be distinguishable from differences in AMD recovery among the individual gradients. To circumvent this problem, an approach was taken which permitted a direct comparison to be made within one gradient.

Random cells were grown for approximately

one-third of a cell cycle (6.5 hr) in the presence of 5-bromodeoxyuridine at a concentration of 5  $\mu$ g/ml ( $1.6 \times 10^{-5}$  M). Nuclei were then isolated in RSB, incubated with AMD-<sup>3</sup>H, washed, and sonicated as detailed in the Materials and Methods. Centrifugation in CsCl (Fig. 3) revealed BUdR-containing, newly-replicated DNA (fractions 12–15) and nonreplicated DNA (17–20) in a ratio of about 1:2.5; however, the newly-replicated DNA contained only about 40% as much actinomycin/OD<sub>260</sub> as the nonreplicated material. In two other experiments the new DNA contained 30% and 45% less actinomycin. The possibility that replacement of DNA thymine by bromouracil interfered with actinomycin binding was ruled out by growing cells in BUdR for a full generation so that all DNA was BUdR-hybrid. There was no difference in the amount of AMD bound by nuclei from these and an equal number of control cells. Furthermore, when these two samples were analyzed in CsCl the banded DNA contained equal amounts of AMD/OD<sub>260</sub> in both cases, demonstrating that the result illustrated in Fig. 3 is not due to a preferential loss of AMD from BUdR-hybrid DNA during the centrifugation.

TABLE I  
Actinomycin Binding in Isolated Nuclei from Synchronized Cells

10 <sup>8</sup> nuclei in:	AMD bound ( $\mu$ mcles)	DNA ( $\mu$ g)	AMD/DNA ( $\mu$ mcles/ $\mu$ g)
Early S	848.0	811	0.104
Late S	743.0	1434	0.052
late S/early S = 50%			

HeLa cells growing at  $2 \times 10^5$  cells/ml were synchronized by a single exposure to 2 mM thymidine for 14 hr, followed by resuspension in fresh medium. At 1 hr (early S) and 7 hr (late S) after release from the thymidine blockade equal numbers of cells were harvested, washed, and homogenized in RSB as detailed in text. Both samples of nuclei were washed three times, adjusted to  $25 \times 10^6$ /ml, and incubated with actinomycin-<sup>3</sup>H (40  $\mu$ Ci/ml or approximately 12  $\mu$ g/ml) in a total volume of 4.0 ml of RSB for 1 hr at 4°C. The nuclei were washed twice in cold RSB and extracted three times in cold ethanol as detailed in text. DNA was measured by the diphenylamine reaction (10 min, 100°C) using calf thymus DNA as a standard.

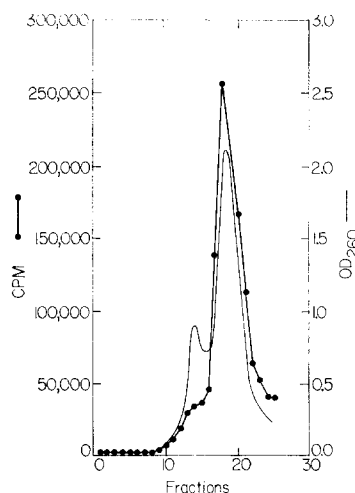


FIGURE 3 Comparison of actinomycin binding in newly-replicated vs. nonreplicated DNA. A 250 ml culture of  $2 \times 10^5$  cells/ml was incubated with 5-bromodeoxyuridine (BUdR) at 5  $\mu\text{g}/\text{ml}$  for 6.5 hr; isolation of nuclei, incubation with actinomycin- $^3\text{H}$ , and preparation for CsCl centrifugation was as described in text (65 rotor, 50,000 rpm, 65 hr). ●—●, actinomycin- $^3\text{H}$ ; — OD $_{260}$ . Two other cultures were grown with and without BUdR for 20 hr and processed as above. In the culture exposed to BUdR all of the DNA banded at fractions 13–15 (BUdR hybrid), while the control DNA was at the usual position (fractions 17–19); however, the amount of actinomycin- $^3\text{H}$  per OD $_{260}$  was the same in both gradients.

#### AMD Binding Capacity of DNA Purified from Synchronized Cells

To investigate whether the decreased AMD binding capacity of chromatin during S and G<sub>2</sub> is related to a structural modification of the DNA itself (e.g. methylation) or to changes in its degree of association with chromosomal proteins, AMD binding was measured in samples of DNA purified from synchronized cells (Fig. 4). Irrespective of cell cycle stage, DNA was found to bind equal amounts of AMD, and with similar kinetics, as is illustrated in Fig. 4 specifically for early S vs. G<sub>2</sub>. The AMD binding changes described for cells and nuclei are thus concerned with a level of organization beyond that of the DNA helix itself.

#### DISCUSSION

The results of this study suggest that dynamic changes occur in chromosomal architecture throughout the G<sub>1</sub>, S, and G<sub>2</sub> portions of the cell

cycle, at an organizational level not resolved by the electron microscope. Transient changes in the submicroscope structure of interphase chromatin have been suggested by a variety of other studies on synchronized mammalian cells. The relative resistance of G<sub>2</sub>-phase Chinese hamster, HeLa, and mouse L5178Y cells to killing by actinomycin (Elkind et al., 1968; Sawicki and Godman, 1971; Doida and Okada, 1972) is probably due to the reduced drug-binding capacity of G<sub>2</sub> chromatin demonstrated in this report. In addition, studies on the survival of synchronized cells following lethal doses of X-irradiation have generally revealed maximal sensitivity in G<sub>2</sub> and mitosis (Elkind et al., 1968; Sinclair and Morton, 1966; Sinclair, 1968), while sublethal doses can affect cells in G<sub>2</sub> selectively (Yamada and Puck, 1961;

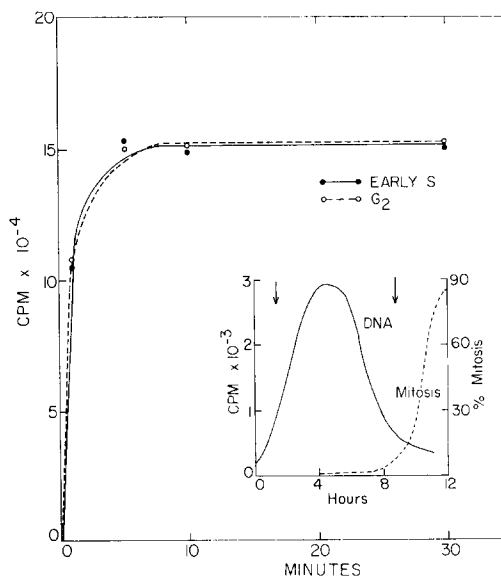


FIGURE 4 AMD binding to purified DNA. At the times indicated in the inset, cells were harvested and DNA purified as detailed in the text. Assays of AMD binding were performed at 4°C using 1  $\mu\text{g}$  of purified DNA and 4  $\mu\text{g}$  of actinomycin; each point on the curves represents the average of two replicates. In parallel assays using 10  $\mu\text{g}$  of DNA (not shown) a ten-fold increase in AMD binding was observed, illustrating that the ligand was present in excess of the DNA under the conditions employed. Preincubation of the DNA samples with 100  $\mu\text{g}/\text{ml}$  of DNase I for 60 min at 37°C resulted in a 95–97% reduction in AMD binding compared to controls incubated without enzyme. ●—●, DNA from early S cells; ○---○, DNA from G<sub>2</sub> cells. Inset: ●—●, DNA synthesis, ○---○, mitotic index (with colchicine added at 6 hr).

Puck and Steffen, 1963). Finally, the analysis of mitotic chromosome aberrations produced by exposure of cells to ionizing radiation in the preceding interphase has revealed that the chromosomes behave as double structures from the end of G<sub>1</sub> onward (Wolff, 1968); the incidence of metaphase chromatid breaks, which are the cytological manifestations of this doubleness, increases as cells are irradiated progressively later in S and G<sub>2</sub>, the periods of reduced AMD binding in the present study. Further combined analyses of AMD binding and radiation responses, within the same synchronized cell populations, will be necessary to determine whether this temporal relationship is approximate or precise.

Measurements of AMD binding during spermiogenesis (Brachet and Hulin, 1969; Darzynkiewicz, Gledhill, and Ringertz, 1969), in phytohemagglutinin-transformed lymphocytes (Darzynkiewicz, Bolund, and Ringertz, 1969) prednisolone-treated thymocytes (Darzynkiewicz and Anderson, 1971), and in heterochromatin vs. euchromatin within the same nucleus (Berlowitz et al., 1969) have all indicated reduced ligand binding in condensed chromatin. While a comparison of random and mitotic HeLa cells would have led to the same conclusion, the use of cells synchronized in S and G<sub>2</sub> reveals that AMD binding decreases progressively over this interval and is not temporally coordinated with chromosome coiling *per se*. It is possible that in certain cases AMD binding is determined by the macromolecular composition of the elementary nucleohistone fibrils themselves, as well as by their three-dimensional packing with respect to one another.

Whether the decreased AMD binding capacity of chromatin during late S and G<sub>2</sub> is functionally related to the mechanism of chromosome coiling is not clear at present. As Prescott has pointed out (1964) the demarcation between G<sub>2</sub> and prophase is defined only by our ability to visualize condensed chromatin by microscopy. Verification of the hypothesis that subtle preparations for chromosome coiling begin in S and G<sub>2</sub> would be aided by determining the AMD binding profiles of cell lines with different cell cycle kinetics, particularly those with long G<sub>2</sub> periods.

#### ADDENDUM

Since this paper was submitted, the concept of changes in chromatin structure during interphase

has been corroborated by experiments with pancreatic DNase (Pederson, T. 1972. *Proc. Natl. Acad. Sci. U.S.A.* **69**:2224.

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