Role of Non-histones in Chromosome Structure

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As part of a study of the role of non-histone proteins in chromosome structure, the synthesis of non-histones associated with interphase chromatin was investigated. Synchronized suspension cultures of HeLa cells were pulse-labeled with [35S]methionine, and chromatin was prepared by mild micrococcal nuclease digestion. Two-dimensional polyacrylamide gel electrophoresis, in addition to one-dimensional electrophoresis, was used to resolve the patterns of incorporation of radioactive label. Significant variations in non-histone synthesis were seen during the cell cycle. A strong correlation was not found between DNA synthesis in mid-S phase and variations in non-histone synthesis.

The non-histone proteins of purified metaphase chromosomes were also characterized by two-dimensional gel electrophoresis and compared to the proteins of interphase chromatin. The pattern of non-histones is not identical with that of interphase chromatin, although a number of major species may be shared by interphase chromatin and metaphase chromosomes. The HeLa nuclear scaffold, the framework that maintains the overall morphology of the interphase nucleus, shows relatively few proteins on two-dimensional gels. The synthesis of nuclear scaffold proteins was quantitated by excising each of 19 proteins from two-dimensional gels and determining the incorporated radioactivity by scintillation counting. Substantial variations in protein synthesis were found, with several species showing changes of about 2-fold in the percentage of incorporation.

Non-histone proteins are major components of metaphase chromosomes and interphase chromatin, yet their precise structural and functional roles are incompletely understood. An approach to uncover a structural role for non-histones in the higher order organization of metaphase chromosomes was to extract histones and other chromosomal proteins and to characterize the residual particles by polyacrylamide gel electrophoresis and electron microscopy (1, 2). The DNA of histone-depleted metaphase chromosomes was found to be arranged into loops by a well defined subset of non-histones, the metaphase scaffold. The significance of a residual chromosomal structure in organizing DNA is now well established (3-13).

The protein framework that maintains the morphology of interphase nuclei has also been studied. Extracting histones and other proteins from nuclei was found to produce residual particles which maintain a high degree of DNA organization (14-16). This result is complemented by experiments showing the interphase genome to consist of looped domains (17, 18). The sites of DNA replication appear to be anchored to the residual protein structure (19, 20). Nuclease digestion has been combined with protein extraction to create a nuclear substructure composed primarily of protein (16, 21-25).

Besides these structural roles with metaphase chromosomes and interphase nuclei, non-histone proteins also have functional roles as regulators of gene expression. In this capacity, non-histones may interact with the nucleosome structure of chromatin to produce specific regulatory effects. The arrangement of histones with DNA to form nucleosomes is well understood (26), but the nature of the involvement of non-histones is less clear.

The experiments described in this paper are concerned with investigating the cell cycle-dependent changes in the synthesis of chromatin-associated non-histones. The experiments are also concerned with the relationship of these proteins to the non-histones of metaphase chromosomes and nuclear scaffolds, residual structures produced by nuclease digestion and extraction of proteins (16). This information is likely to be crucial to an understanding of the structural and functional changes in the genome through interphase and into mitosis.

To obtain a high resolution separation of the proteins of chromatin and chromosomes, two-dimensional polyacrylamide gel electrophoresis has been applied. Previous studies have used the high resolution of two-dimensional gels to examine polypeptide synthesis in whole cells and nuclei (27, 28) and to survey HeLa non-histone proteins (29). One-dimensional polyacrylamide gels have shown different profiles for acidic chromosomal protein during G1, S, and G2 (30, 31).

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization—HeLa S3 cells (ATCC CCL 2.2) were maintained in suspension culture at 37 °C in minimum essential medium (Joklik-modified) supplemented with 10% newborn calf serum (Grand Island Biological Co.). Synchronized cells were obtained with a double block of thymidine (32) or hydroxyurea (33). A typical experiment, thymidine was added to a suspension culture of HeLa cells to a final concentration of 2 mM or 5 mM. After 16 h, the block was released by washing the cells in normal medium, and, after a further 9-h incubation, the cells were again blocked with thymidine for 16 h. Releasing the culture from the second block gave a population of cells synchronized at the G1/S boundary. The degree of synchrony was routinely estimated by measuring the incorporation of [3H]thymidine into DNA. A peak of incorporation was found at 5 h after release from the G1/S boundary, a result similar to that previously reported (30). The degree of synchrony was also determined by counting the number of mitotic cells and the total concentration of cells. To aid the identification of mitotic cells, portions of synchronized cultures were withdrawn and resuspended in hypotonic buffer (10 mM NaCl, 10 mM Tris, pH 7.4, 5 mM MgCl2) prior to counting.

The maximum fraction of mitotic cells was found at 11 h after release from G1/S, while a doubling of the concentration of cells was found at 12-13 h.
 Chromatin Preparation—To measure the synthesis of chromatin- 
asociated non-histones during the cell cycle, portions of cultures 
synchronized with double blocks of thymidine or hydroxyurea were 
withdrawn at intervals of 4 h following release from the G_{1}/S bound- 
ary. Each portion was concentrated by centrifugation to give 10^6 cells in 1 ml, and 0.5-1.0 mg of [3H]methionine was added. The cells were in 
normal minimum essential medium or in a 1:1 mixture of normal 
minimum essential medium and medium lacking methionine. The 
cells were labeled for 1 h at 37 °C in a gyratory water bath, after 
which the cells were rapidly cooled on ice. Nuclei were then prepared (16). The cells were washed in a buffer of 10 mM NaCl, 10 mM Tris, pH 7.4, 5 mM MgCl_2, 0.1 mM phenylmethylsulfonl fluoride at 0 °C, 
and resuspended in the buffer at a concentration of about 10^7/ml. After 
leaving the cells for 30 min in the hypotonic buffer, the cells 
were disrupted with a Dounce homogenizer, following the addition of 
0.5% Nonidet P-40 and 0.1% sodium deoxycholate. The nuclei were then 
centrifuged and resuspended at a concentration of about 2 x 10^8 nuclei/ml. Fig. 1 shows the protein species found with preparations of isolated nuclei. For the sample in lane b, only the non-ionic detergent Nonidet P-40 was used in isolating nuclei, while for the sample in lane c, sodium deoxycholate was also included. The inclusion of sodium deoxycholate is useful in removing residual cy- 
toskeletal material which sometimes remains attached to the nuclei. 
Fig. 1 demonstrates that low concentrations of deoxycholate do not 
have a detrimental effect upon nuclear protein composition. The 
same pattern of species is seen in lane c as in lane b.

Chromatin was obtained by mild digestion of nuclei with micrococcal nuclease (34). Nuclei were treated with micrococcal nuclease at 
a concentration of 15 units/ml. The supernatant of unfractonated chromatin was used without further purification or was fractionated 
on density gradients of 5-30% sucrose (35). The gradients (5 ml) were 
prepared in 10 mM Tris, pH 7.4, 1 mM EDTA, 0.1% Nonidet P-40, 
and, after layering 100 µl of chromatin on each gradient, centrifugation 
was for 17 h at 33,000 x g (20,000 rpm). Similar experiments were 
carried out with chromatin labeled with [3H]thymidine to demon- 
strate that the protein and DNA peaks coincide.

Metaphase Chromosome Preparation—HeLa metaphase chro- 
mosomes were isolated by procedures in which the chromosomes are 
maintained in a condensed state with divalent cations or polyamines 
while the mitotic cells are disrupted in a Dounce homogenizer (36). To obtain an adequate number of mitotic cells, suspension cultures 
were arrested in mitosis by adding 0.2 µg/ml of colchicine for 12-16 h. The cells were resuspended in a buffer containing 50 mM NaCl, 5 mM 
HEPES, pH 7.4, 5 mM MgCl_2, 0.5 mM CaCl_2, 0.1 mM phenylmethyl- 
sulfonl fluoride. An alternate, polyanionic-containing buffer is com-
Cell Cycle Variations in Non-histone Synthesis

Non-histone species are seen to be associated with chromatin cells. Columns a, 0- to 1-h samples. Cells at 0-1 amide gels, while A double thymidine block was used to obtain synchronized dimensional gels, which are described below, resolve more labeled at than . Variations in the synthesis of non-histones for synchronized cells pulse-labeled with micrococcal nuclease and was purified on sucrose density gradients. At least obtained by digestion with micrococcal nuclease and was purified on ["%]methionine that are found for non-histones labeled at . The pattern of ["S]methionine in- mates the true number because of overlapping of bands. Two-dimensional gels, which are described below, resolve more than 300 species.

Fig. 3 reveals the significant variations in the incorporation of ["S]methionine that are found for non-histones labeled at 0-1 h and 12-13 h after release from the G1/S boundary. Variations in the synthesis of non-histones for synchronized cells pulse-labeled at 4-5 h and 8-9 h are intermediate to the differences found at 0-1 h and 12-13 h. There does not, therefore, appear to be a strong correlation between DNA synthesis, which peaks at about 5 h after G1/S, and changes in non-histone synthesis. The pattern of ["S]methionine incorporation from cells labeled at 16-17 h is also similar to the 0- to 1-h samples. Cells at 16-17 h after G1/S have progressed into G1 of the next cell cycle, and thus protein synthesis in G1 is seen to resemble that in early S phase.

Densitometer scans of fluorograms of interphase chromatin non-histones from synchronized cells labeled with ["S]methionine at (a) 0-1 h and (b) 12-13 h after G1/S. Electrophoresis was in gels of 8% acrylamide. (The fluorograms are shown in Fig. 3, d and e.)

Synthesis of Interphase Non-histones: Two-dimensional Gel Electrophoresis—Although one-dimensional electrophoresis is useful for directly comparing different chromatin samples in adjacent lanes of slab gels, two-dimensional electrophoresis provides a higher resolution separation of individual protein species. Fig. 5 contains two-dimensional gels of chromatin from cells labeled with ["S]methionine at (a) 0-1 h and (b) 12-13 h after release from the G1/S boundary. The cells were synchronized with a double thymidine block. The chromatin samples were not fractionated on sucrose gradients, but the patterns with fractionated chromatin are similar. The nonequilibrium procedure (39) was used to separate proteins according to their charge in the first dimension, while electrophoresis in 8% SDS-polyacrylamide gels was used in the second dimension.

The complexity of the non-histone composition of HeLa chromatin is apparent from Fig. 5. Over 300 different species can be counted. Some additional proteins of lower molecular

\[\text{Molecular Weight (x10}^{-3}]\]

\[\begin{array}{cccc}
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\(\text{pH} 7\)
weight can be observed when samples are electrophoresed in SDS gels containing a higher percentage of acrylamide, although the preponderance of chromatin non-histones have molecular weights greater than 30,000. The nonequilibrium technique separates basic as well as acidic proteins, and it can be seen from Fig. 5 that both of these classes of proteins are well represented among the non-histones of interphase chromatin. The majority of species, however, are acidic. Many changes can be observed in the degree of incorporation of [\textsuperscript{35}S]methionine when comparing the electrophoretic patterns for 0-1 h and 12-13 h chromatin. But while the changes in non-histone synthesis are significant, they are not dramatic. As was also observed with one-dimensional SDS-polyacrylamide gels, non-histone synthesis at 4-5 h, 8-9 h, and 16-17 h shows patterns intermediate to those at 0-1 h and 12-13 h.

Experiments were undertaken to determine the effect of DNA replication inhibitors upon non-histone synthesis. DNA replication was inhibited in one portion of a synchronized culture by adding 5 mM thymidine or 1 mM hydroxyurea. At 4-5 h after release from G\textsubscript{1}/S, both the uninhibited and inhibited portions were labeled with [\textsuperscript{35}S]methionine, chromatin was prepared, and the chromatin-associated non-histones were resolved on two-dimensional gels. The patterns of incorporation of label were similar whether or not the DNA replication inhibitor was present, although the overall level of protein synthesis was reduced by 50% in the presence of inhibitor.

Pulse-chase experiments have been carried out to examine the later fate of the incorporated label during the cell cycle. Synchronized cells, prepared with a double thymidine block, were pulse-labeled with [\textsuperscript{35}S]methionine for 0-1 h after release from G\textsubscript{1}/S and then resuspended in fresh medium, free of radioactive label, for an additional 4 or 7 hours. Chromatin was obtained, and the associated non-histones were electrophoresed on two-dimensional gels. Fig. 6 shows a two-dimensional gel for such an experiment and reveals differences in the distribution of label among chromatin non-histones compared to samples that were not chased (Fig. 5). A number of factors could be responsible for this result, including differences in the stability of association of non-histones with chromatin, in the rates of synthesis, in post-translational modifications, and in the rates of degradation.

The experiments presented in Figs. 2 to 6 used cells synchronized with a double thymidine block, but a number of experiments used samples prepared with a double hydroxyurea block. Two-dimensional gels of pulse-labeled chromatin derived from hydroxyurea-synchronized cells showed results similar to those with thymidine-synchronized cells.

**Relationship of Non-histones of Metaphase Chromosomes and Interphase Chromatin**—The non-histone proteins associated with purified HeLa metaphase chromosomes have been characterized and compared to the proteins of interphase chromatin by two-dimensional gel electrophoresis. Fig. 7a presents a two-dimensional gel of metaphase chromosomes isolated in a pH 7.4 buffer containing divalent cations (Mg\textsuperscript{2+}, Ca\textsuperscript{2+}). The chromosomes were purified by sedimentation into a density gradient of Metrizamide. a, HeLa metaphase chromosomes. b, mixture of metaphase chromosomes and interphase chromatin. In b, the positions of major non-histones of metaphase chromosomes (□) and interphase chromatin (○) are indicated.

![Fig. 6. Two-dimensional polyacrylamide gel of a pulse-chase experiment with chromatin non-histones from synchronized cells. Cells were labeled with [\textsuperscript{35}S]methionine at 0-1 h after release from the G\textsubscript{1}/S boundary, and, after removing the isotope, the cells were incubated for an additional 7 h. Chromatin was prepared, and the associated non-histones were separated on two-dimensional gels by the nonequilibrium procedure (39) combined with 8% SDS-polyacrylamide slab gels. The proteins indicated by circles show large changes in the distribution of label among chromatin non-histones compared to control samples (Fig. 5a).](image-url)
chromatin before electrophoresis. The result of a mixing experiment is shown in Fig. 7b. The positions of some of the major proteins of metaphase chromosomes and interphase chromatin are indicated on the figure. Such experiments confirm the observation that a number of the major proteins are distinct to chromosomes or to chromatin. Although this experiment uses proteins labeled with a radioactive isotope, the same result is found using gels stained with Coomassie brilliant blue or a silver stain. The silver-stained patterns of interphase chromatin non-histones resemble the fluorograms of Figs. 5 and 7a, respectively.

Synthesis of Nuclear Scaffold Proteins—The nuclear scaffold is a substructure that maintains the general morphology of the nucleus and that may be involved in the long range organization of chromatin (16). To characterize nuclear scaffold proteins, samples were electrophoresed on two-dimensional gels (Fig. 8). The samples were obtained by DNase I (100 μg/ml) treatment and extraction with 2 M NaCl at pH 7.4. Only a relatively few proteins are seen on the two-dimensional gels. One group has molecular weights of around 65,000-70,000, and some of these proteins are probably identical with the peripheral lamina proteins (22). Comparing the gels of interphase chromatin non-histones (Fig. 5) with Fig. 8 demonstrates that a number of scaffold proteins are distinct from chromatin non-histones.

The synthesis of nuclear scaffold proteins during the cell cycle was quantitated by directly measuring the [35S]methionine radioactivity incorporated into a majority of the major and minor proteins (Table I). Cells were synchronized with double thymidine blocks, and portions of the synchronized cultures were radioactively labeled at 0-1 h, 4-5 h, 8-9 h, 12-13 h, 16-17 h, and 20-21 h after release from the G1/S boundary. Nuclear scaffolds were obtained, and the scaffold proteins were resolved by electrophoresis on two-dimensional polyacrylamide gels. The proteins were located by staining with Coomassie brilliant blue. Stained areas corresponding to each of 19 proteins were cut from gels and dissolved by heating in H2O2 before determining the radioactivity in a scintillation counter.

These specific changes measured for the synthesis of nuclear scaffold proteins confirm the observations with two-dimen-

![Fig. 8. Nuclear scaffold proteins electrophoresed on a two-dimensional gel and stained with Coomassie brilliant blue.](image)

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*a* The times denote the 1-h periods of labeling with [35S]methionine after release of synchronized cells from the G1/S boundary. The position in the cell cycle is given in parenthesis.

*b* The uncertainty in the average values for major species such as proteins 2 and 10 is estimated to be ±3%, while the uncertainty for minor species such as proteins 1 and 12 is estimated to be ±20%.
A major purpose of this investigation was to use two-dimensional gels to determine the variations during the cell cycle in the synthesis of non-histones associated with interphase chromatin. Such variations may be involved in changes in the structural organization of the HeLa genome during interphase. The non-histones of interphase chromatin were also compared to the proteins of isolated metaphase chromosomes. In mitosis the genome undergoes its greatest organizational change to form the characteristic structural features of metaphase chromosomes. Understanding the differences in non-histones from interphase to metaphase may help to reveal the interactions which are responsible for the condensation of metaphase chromosomes. Another purpose of these experiments was to determine whether nuclear scaffold proteins are unique species or are also found as major proteins of chromatin.

Chromatin was prepared by micrococcal nuclease digestion of isolated nuclei after labeling synchronized cells at 0-1 h, 4-5 h, 8-9 h, 12-13 h, and 16-17 h after release from the G1/S boundary. Electrophoresis on one-dimensional SDS-polyacrylamide gels revealed significant differences in non-histone synthesis, particularly when comparing early S phase (0-1 h after G1/S) and a period close to mitosis (12-13 h after G1/S). The peak of DNA synthesis occurs at around 5 h after release from G1/S, but this burst of nucleic acid synthesis is not correlated with a substantial change in non-histone synthesis. 8-9 h samples also show an intermediate pattern of protein synthesis. By 16-17 h after G1/S, the synchronized cells have progressed through mitosis and into G2 of the next cell cycle, and protein synthesis at 16-17 h also has a pattern between that of 0-1 h and 12-13 h samples.

Two-dimensional polyacrylamide gel electrophoresis extends these results by providing a higher resolution separation of proteins. On two-dimensional gels, the patterns of incorporation of [35S]methionine show significant changes during the cell cycle, but, as was the situation with one-dimensional gels, abrupt, large-scale alterations in protein synthesis are not observed. Instead, a progressive modulation of protein synthesis is found which shows the greatest difference when comparing 0-1 h and 12-13 h samples.

Isolated metaphase chromosomes were also seen to have a complex composition of non-histone proteins. Over 280 species, both basic and acidic and possessing a wide range of molecular weights, could be counted on two-dimensional gels. The pattern of proteins is not identical with that of interphase chromatin. A reason for this could be that a number of metaphase non-histones are structural components of regions unique to metaphase chromosomes, such as the kinetochore, and would, therefore, not have a counterpart among interphase chromatin non-histones. In addition, proteins that are involved in maintaining the condensed state of chromosomes may be found as components of metaphase chromosomes. And since metaphase chromosomes are effectively inactive in gene expression, proteins involved in switching from the active to inactive states of the genome could also account for the differences between metaphase chromosomes and interphase chromatin.

In addition to chromatin, another major component of the interphase nucleus is the nuclear scaffold. The nuclear scaffold is the structural framework which maintains the large scale features of nuclear morphology. The framework helps to compartmentalize the interphase genome, and, since structure is intimately related to function, this higher order constraint upon the genome is also likely to be important in gene expression. The protein composition of nuclear scaffolds revealed by two-dimensional electrophoresis is much less complex than either chromosomes or chromatin. Some of these proteins are distinct from major components of chromatin. This is to be expected in view of the separate nature of the nuclear scaffold.
Because of the relatively small number of proteins associated with the nuclear scaffold, this substructure was chosen to quantitate changes in the incorporation of \[^{35}\text{S} \]methionine during the cell cycle. Directly determining the incorporated radioactivity for each of 19 proteins showed variations of around 2-fold for several species. The changes, for the most part, represented gradual increases or decreases in incorporation through the cell cycle. Some anomalies were seen for samples labeled at 12-13 h (M/G1). A correlation was not found between DNA synthesis, which peaks at 5 h after release from G1/S, and the synthesis of nuclear scaffold proteins.

These results establish the basis for further experiments that have the ultimate aim of correlating changes in non-histones with changes in the structure and function of the interphase and metaphase genome.

REFERENCES