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Proposed Coupling of Chromatin Replication, Hormone Action, and Cell Differentiation[1]

Gerald C. Mueller,[2] Kazuto Kajiwara, Uh Hee Kim, and Joyce Graham

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Abstract

DNA replication in isolated nuclei is highly dependent on the availability of soluble proteins from the cell cytoplasm. Activity is distributed nonrandomly among the different proteins, and the range of proteins that are required for optimal DNA replication varies with the fractions of DNA being replicated. Support of DNA replication has been correlated with the uptake of these proteins by nuclei and their integration into an immature form of the newly replicated chromatin; the latter has been shown by density analysis to be richer in protein content than the bulk of nonreplicating chromatin. Pulse labeling of DNA in living cells has revealed that a similar protein-rich chromatin is formed as an intermediate in chromatin replication *in vivo;* however, this form rapidly matures by the exclusion of proteins. The dependency of DNA replication on the presence of soluble cytoplasmic proteins and the physical association of these entities with newly replicated chromatin prompt the proposal that availability of specific proteins may play an important role in determining the ultimate genetic expressability of the matured chromatin and thus the cell phenotype. The finding that dexamethasone, a steroid that regulates the expression of several genes and directs the differentiation of certain cells, can modify the uptake of proteins in isolated nuclei is in accord with this hypothesis.

Introduction

The structure and function of organized tissues depend on the balance between replication and differentiation of the constituent cell types. In this situation each cell responds as a unit to factors in its environment that directly influence ongoing catalytic processes or alter the expression of genes, leading to the production of proteins that are essential for these processes or structural aspects of cell life. The range of responses, and in each case the contribution of the cell to the tissue, is characterized by the phenotype of the cell. Both the probability of its replication and demise and its functional contribution to the tissue are determined by the spectrum of genes that exist in the on state or potentially inducible state. A tremendously important aspect of tissue growth is that the constituent cells are continually and progressively changing their phenotype in response to hormonal, nutritional, physical, and pharmacological stimuli. This situation is dramatically revealed in the hyperplastic response of tissues like the uterus or mammary gland to sequential stimulation by hormones. In such cases certain cells replicate rapidly and exhibit unique functions, but in so doing they irreversibly change their character so that their requirements for and responses to factors in the local environment are grossly changed. When the hormonal support is withdrawn, the cells may pass into a terminal aspect of their life or into other states, which are quite different from those of the starting cells. These cells have undergone cell differentiation, and the spectrum of their inducible genes has been changed dramatically. This progression is fundamental to the maintenance of organized tissue growth in all settings, and aberrations of the fundamental processes may play an important role in growth dyscrasias, such as cancer and tissue aging. An understanding of the molecular processes that operate in cell differentiation is a major goal of the studies in our laboratory. In this presentation we describe certain aspects of chromatin replication, which appear to provide a molecular basis for changing chromatin character and may account in part for the alterations of gene expression entailed in cell differentiation. Within the proposed concepts lies a possible explanation for the frequently observed coupling of cell replication and cell differentiation.

Some Molecular Aspects of DNA and Chromatin Replication

Cells are frequently described as progressing around a cell cycle as portrayed in Chart 1. In the tissue setting, each cell senses continually for that stimulus or combination of stimuli that will activate the genes for the production of proteins required for DNA and chromatin replication. For this paper it is proposed that this activation event operates much like the activation of any other major genetic operon, the difference being that it locks a cell into a tightly coupled sequence of molecular processes in which the chromatin (*i.e.*, the genetic material itself) is replicated and distributed to daughter cells. The limited evidence available suggests that the initial mitogenic stimulus for each cell is characteristic of the cell phenotype and is sensed by receptor-like states of the cell membrane. Perturbation of the latter leads directly or indirectly (it is not yet clear) to the activation or release of some component (presumably a protein) that translocates to the nucleus and activates the replication operon. The events that ensue are tightly coupled in eukaryotic cells and to a large extent appear to be paced by the progress of DNA replication.

Before proceeding to the molecular and enzymatic steps in chromatin replication, it is well to reflect briefly on the structure of chromatin and the complexity of the problem of its replication. Through the rapid developments of the

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[2] Recipient of a USPHS Research Career Award. To whom requests for reprints should be addressed.

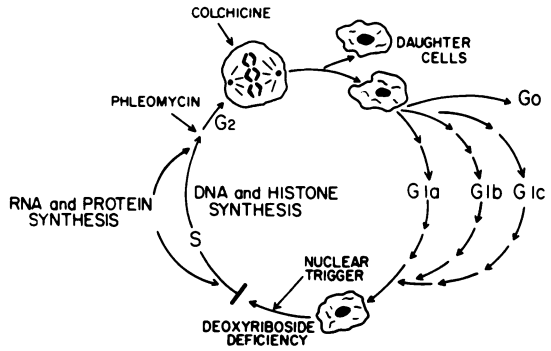

Chart 1. Diagram of a cell cycle. The replication of animal cells is a highly ordered sequence of molecular events requiring the expression of specific genetic information at certain steps in the cycle. The S phase, an interval lasting 6 to 7 hr in many cells, is concerned with the synthesis of a complete complement of DNA and histones; progression through this interval requires the timely synthesis of RNA as well as DNA and proteins. These processes are tightly coupled. The G_2 interval, including mitosis and cell division, requires approximately 2 to 3 hr and is concerned with the formation of newly synthesized chromatin into chromosome pairs and the distribution of the condensed chromosomes into the daughter cells. Progression into the G_2 interval requires the completion of DNA synthesis and the antecedent synthesis of RNA and protein. In HeLa cells, phleomycin blocks the cell cycle near the end of the DNA synthesis period. Colchicine and other agents active against the microtubules block the progression of cells through metaphase. Cells with different cycle times vary mainly in the duration of the G_1 interval and are depicted in the diagram as G_{1a}, G_{1b}, G_{1c}, and G_0. During this interval the cell expresses the phenotypic character of its particular differentiated state. Depending on intracellular and extracellular factors, cells progress through G_1 and become triggered for nuclear replication; this involves activating genes for DNA replication and the synthesis of the component of DNA replicase.

last four years, we have come to know that nucleochromatin is composed of a linear array of nucleosomes in which 190 to 210 base-pair lengths of DNA are wrapped around 2 tetrameric assemblies of histones H2A, H2B, H3, and H4. This string of beads is supercoiled in many regions by the interaction of H1 histones, which complex the free DNA lying between the linearly distributed nucleosomes (5). In most eukaryoic cells the gross mass of histones is roughly equivalent to the DNA mass. In addition, there is a quantity of nonhistone proteins associated with nucleochromatin; the amount and molecular character of these proteins vary with the cell phenotype and functional activity of the cell. The exact location of the nonhistone proteins amid the chromatin structure is not well known at this time, except that certain of these proteins are nonrandomly distributed (8).

Radioautographic analysis of replicating DNA (1) has shown us that DNA replication in the chromosome of the eukaryotic nucleus proceeds simultaneously from multiple sites. The activation of these sites is a highly ordered process that requires the continued synthesis of DNA, RNA, and protein (7). A single replicon (*i.e.*, the unit of chromosomal DNA replication) corresponds to 30×10^6 to 60×10^6 daltons of DNA, which is equal to 100×10^5 to 200×10^5 base pairs distributed between 500 and 1000 nucleosomes. The DNA coursing over the nucleosomes of a single replicon exhibits 10×10^3 to 20×10^3 turns of the double helix, in addition to those involved in the supercoiling of the compound chromatin structure seen in interphase nuclei (5). A simple topographical assessment of the problems inherent in replicating such a structure fills one with appreciation for the role of macromolecular interactions in ordering and directing the chromatin replication process. It seems likely that the same forces that operate in the assembly and processing of viral and subviral structures play a role in the replication and construction of chromatin. In contradistinction to the situation in mature viruses, some of the genes of the linear structure of chromatin, however, must be packaged in a way that allows them to be accessible for genetic expression, a situation that underlies the differentiation state of the cell.

In an attempt to gain some insight into these problems, our laboratory has been investigating molecular mechanisms for DNA and chromatin replication in isolated nuclei of S-phase-synchronized HeLa cells (2, 3). The nuclear system that has been developed continues to replicate DNA from sites that were active in the living cells. When appropriately supplemented, the nuclei replicate approximately 5% of nuclear DNA before degenerative processes dominate the system. The success of the system requires that nuclei come from S-phase cells with a complement of DNA replicase components and that the reaction mixture provides the 4 deoxynucleoside triphosphates, ATP, Mg^{2+}, and the proper ionic strength. In addition, there is a remarkable requirement for the soluble proteins of the HeLa cell cytoplasm. In the absence of these proteins, DNA replication ceases within 10 min and is restricted to 10% or less of the complete system; in their presence DNA replication continues for more than an hour (2, 3). The newly replicated DNA and chromatin appear to have many properties in common with newly replicated chromatin of living cells.

Studies of DNA chain growth in the complete nuclear system show that the process is a discontinuous one involving the direct extension along one template strand while repairing back with short segments on the opposite template strand (Chart 2). With time, the short intrareplicon DNA segments are ligated to form completed replicons that are subsequently ligated to contiguous replicons. In the absence of the soluble cytoplasmic proteins, not only is the amount of DNA that is replicated very small, but also the individual segments remain short (6). Clearly the soluble proteins support both DNA chain growth and the intrareplicon initiation process.

A large effort has been directed into the fractionation of the active components of the soluble cytoplasmic fraction.

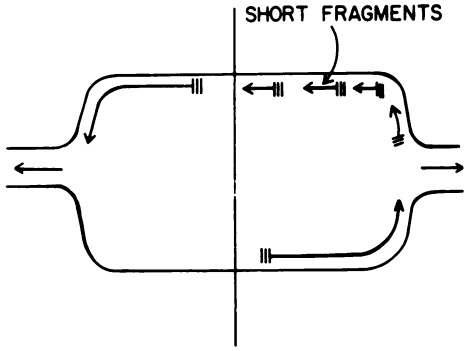

Chart 2. Diagram of DNA chain growth within a replicon. Starting from a central initiation site, DNA replication proceeds bidirectionally within the replicon by direct extension along one template strand and the production of short repair-back DNA segments along the other template strand (6). Ribonucleotide chains are transitorily attached to the 5′-ends of the DNA segments and may be involved in their initiation. With time, the short chains are ligated together to form a completed replicon that is subsequently ligated to an adjacent replicon.

The active components are clearly proteins that are separable by the usual chromatographic and electrophoretic approaches. DNA replication-supporting activity, however, is widely distributed among the many proteins. Each fractionated component accounts for only a small part of the activity of the total cytoplasmic fraction; however, they appear to be additive and in certain cases synergistic in their effects. In addition, DNA replication of early S-phase nuclei appears to require a different combination of these components from that required for the replication of DNA in late S-phase nuclei. These studies have suggested that the individual soluble proteins of the cytoplasm may play some structural role in chromatin replication rather than functioning enzymatically in DNA polymerization.

To explore this situation further, we have investigated the possibility that some of the cytoplasmic proteins might be incorporated into the structure of newly replicated chromatin. For this purpose labeled cytoplasmic proteins were prepared by growing cells in [^{3}H]leucine; these were used as the cytoplasmic protein support in the nuclear system. At the end of the DNA replication interval, the nuclei were separated from the reaction mixture and washed with the buffered 0.9% NaCl solution to remove easily-dissociable labeled cytoplasmic proteins. Up to 2% of the added radioactive cytoplasmic proteins were retained in the nuclei. Electrophoretic analysis of the nuclear proteins in sodium dodecyl sulfate-acrylamide gels revealed that many different proteins had been taken up, that the uptake was nonrandom, and that the uptake by early and late S-phase nuclei differed significantly.

Encouraged by these results we analyzed the nuclei to ascertain whether the labeled proteins were associated with chromatin. For this purpose the labeled nuclei were mechanically sheared, and the chromatin was fixed with formalin according to the procedure of Jackson and Chalkley (4) and sedimented in a CsCl density gradient. A significant fraction of the labeled cytoplasmic proteins was found to be associated with the chromatin peak. As shown in Chart 3, the peak of cytoplasmic proteins was displaced to the light side of the bulk chromatin peak, which was marked by [^{14}C]thymidine labeling in the living cells. This distribution corresponded exactly to the distribution of the newly replicated DNA. It would appear from this result that newly replicated chromatin in isolated nuclei is richer in protein content than the bulk of the chromatin and that the extra proteins have their origin in the soluble proteins of the cytoplasmic fraction.

For testing whether this was also a property of newly replicated chromatin in living cells and for excluding possible artifacts of the nuclear system, the chromatin of cells pulse-labeled *in vivo* with [^{3}H]thymidine was prepared and centrifuged in CsCl density gradients. In accord with the results from isolated nuclei, the newly replicated chromatin of living cells also centrifuged to the protein-rich side of the chromatin band; however, when the chromatin was allowed to mature in the living cell, it rapidly became distributed throughout the chromatin peak. At 37° in the living cells, this redistribution was achieved during a 5-min maturation interval; the process, however, was significantly slowed and easily followed when the experiments were carried out at 27° (Chart 4).

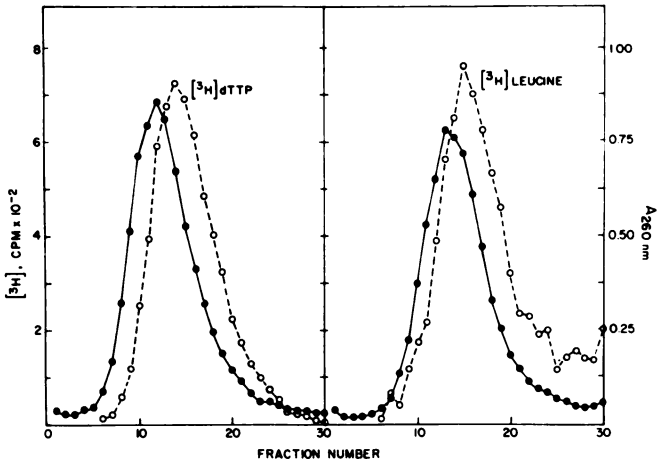

Chart 3. Density of newly replicated chromatin. Formalin-fixed chromatin was prepared (4) from nuclei that had synthesized their newly replicated chromatin *in vitro* in the presence of [^{3}H]dTTP- or [^{3}H]leucine-labeled cytoplasmic proteins. The chromatin was centrifuged in density gradients of CsCl and guanidine:HCl. Fractions were collected from the bottom of tubes; the heaviest fraction = 1. *Solid lines*, sedimentation of the DNA of the total chromatin as indicated by $A_{260\ nm}$ or [^{14}C]thymidine-labeled cellular DNA; *dashed lines*, sedimentation of the newly replicated DNA (*e.g.*, [^{3}H]dTTP) and the associated labeled proteins (*e.g.*, [^{3}H]leucine), respectively. Note that the chromatin that was replicated *in vitro* is lighter than the bulk of the chromatin.

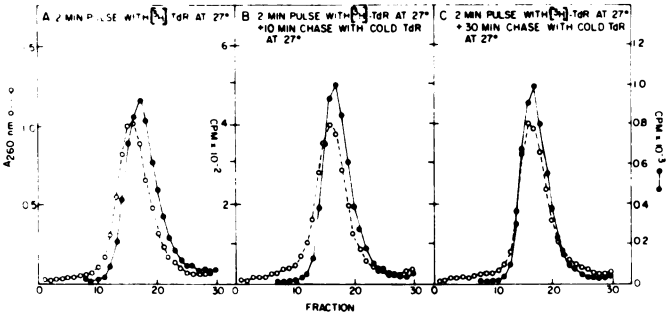

Chart 4. Maturation of chromatin in living cells. HeLa cells were pulse labeled with [^{3}H]thymidine (*[^{3}H]TdR*) for 2 min at 27° (*A*); cells were then allowed to grow for 10 min (*B*) or 30 min (*C*) at 27° in the presence of a diluting pool of unlabeled thymidine (*TdR*). Nuclei were isolated at the indicated times, and chromatin was prepared, and fixed with formalin (4), and centrifuged in a CsCl density gradient. Fractions are collected from the bottom of the tubes with Fraction 1 having the highest density. *Dashed lines*, location of the bulk chromatin in the gradient ($A_{260\ nm}$); *solid lines*, location of the pulse-labeled DNA in the chromatin pattern at different times after replication. Note that the newly replicated DNA (chromatin) is initially lighter than the bulk of the chromatin, but that it shifts with time of maturation to a similar density distribution.

In preliminary attempts to show that the uptake of cytoplasmic proteins into the new chromatin of S-phase nuclei might be modulated by factors known to influence chromatin function, we have tested several steroid hormones for possible activity. In these studies the S-phase nuclei, under conditions that support DNA replication, have been exposed to a 1:1 mixture of [^{3}H]leucine-labeled HeLa cytoplasmic proteins and rabbit uterine cytosol in the presence and absence of the steroid hormones. At the end of the incorporation period, the nuclei were isolated, washed to remove loosely associated proteins, and subjected to a 30-sec digestion with micrococcal endonuclease according to the procedure described by Tata and Baker (9). As shown by these investigators, this treatment releases open and

transcriptionally active chromatin from nuclei; in our laboratory it has been shown that the procedure also releases newly replicated DNA:protein complexes preferentially. The released chromatin fragments from such experiments have been analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis for their representation of labeled cytoplasmic proteins. As shown in Chart 5, the pattern of labeled cytoplasmic proteins contained in the material released by the nuclease treatment is different when dexamethasone is present during the initial uptake interval. This pattern is, however, a very complex one and subject to considerable variation from experiment to experiment. At the present time our conclusion must be limited to the statement that dexamethasone can modify significantly the uptake of cytoplasmic proteins into chromatin under conditions that support chromatin replication. Much more work, however, remains to be done to refine and understand the system.

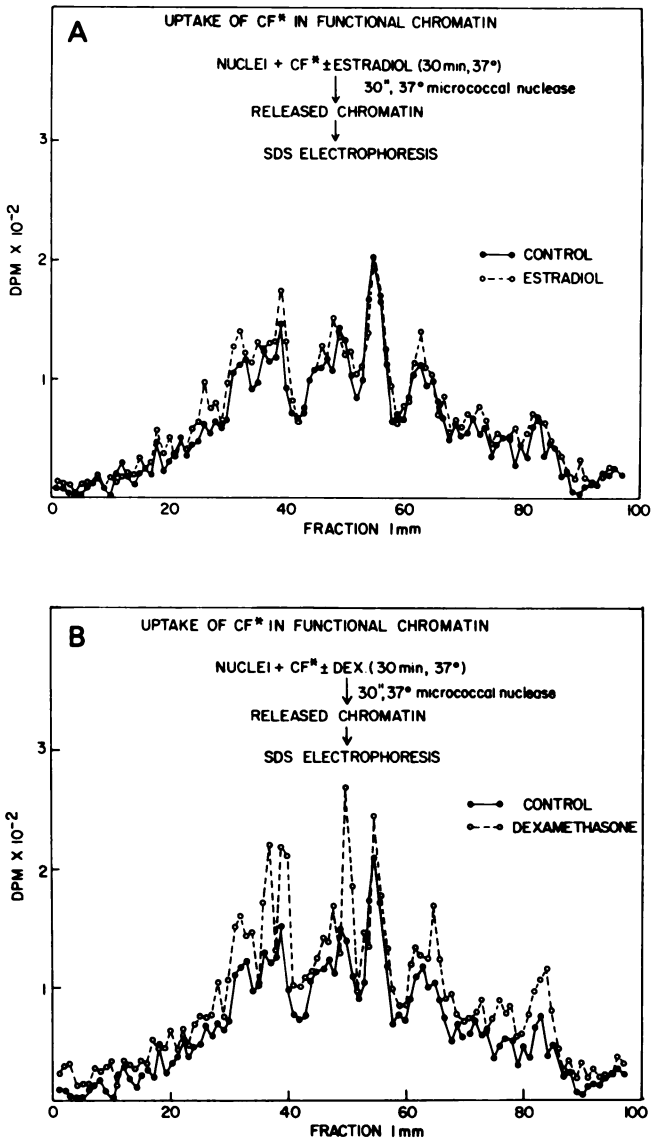

Chart 5. Effects of dexamethasone (*DEX*) on the uptake of cytoplasmic proteins into chromatin. Nuclei from S-phase-synchronized HeLa cells were incubated for 30 min at 37° under conditions that support DNA replication (2) in the presence of a 1:1 mixture of [^{3}H]leucine-labeled HeLa cell cytoplasmic proteins (*CF**) and rabbit uterine cytosol. The rabbit uterine cytosol was preincubated for 1 hr in the presence or absence of 10^{-6} M dexamethasone at 4° prior to use in the protein uptake studies with nuclei. After the labeling the nuclei were washed to remove easily dissociated cytoplasmic proteins and subjected to a 30-sec digestion at 37° with micrococcal nuclease according to the procedure described by Tata and Baker (9). The proteins of the released chromatin fragments were subjected to electrophoresis in sodium dodecyl sulfate (SDS)-acrylamide gels (8.5%) and 1-mm gel fractions analyzed for the presence of radioactivity. Data are reported as dpm × 10^{-2}/fraction.

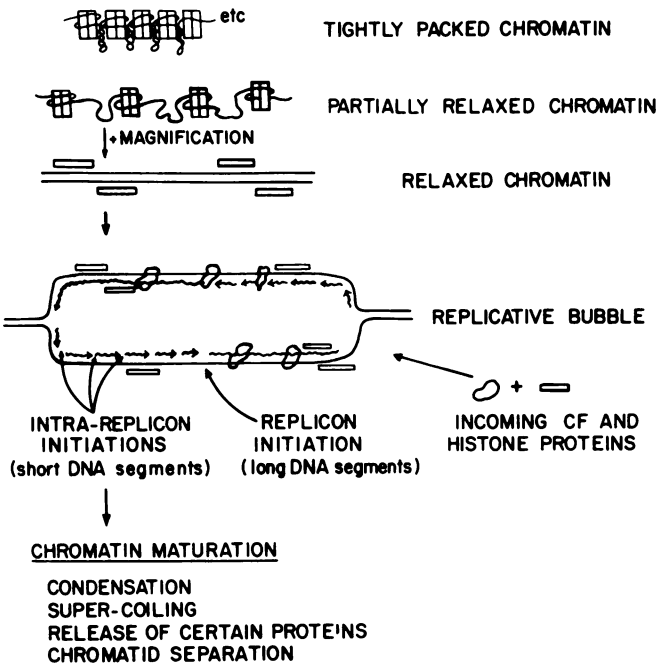

Chart 6. Proposed scheme of chromatin replication. Interphase chromatin exists as a highly supercoiled and aggregated fibrous structure in which the DNA appears to be wound around 2 tetrameric units of histones H2A, H2B, H3, and H4. In the tightly packed state, histone H1 is considered to complex the DNA between the nucleosomes. Preparation for replication appears to require relaxation and uncoiling of specific segments of chromatin at the appropriate time. Although the processes involved in this change are not yet established, a modification of the relationship of histone H1 to the DNA fiber, the unpairing of the histone tetramers, and the enzymatic relaxation of the DNA fiber are proposed. DNA replication is initiated at multiple sites within the relaxed chromatin structure and proceeds bidirectionally within a replication bubble: one strand, by direct extension from the replicon initiation site; and the opposing strand, by a multiple repair-back process that involves the synthesis of short DNA chains that are subsequently ligated together. Incoming proteins, both histones and nonhistones, are required for both the intrareplicon initiation of DNA synthesis and the packaging of the newly replicated DNA. The extended newly replicated chromatin is protein rich. Chromatin maturation involves a process of condensation and recoiling, which releases certain of these cytoplasmic proteins (*CF*) and returns the chromatin to the interphase state.

Chromatin Replication Process—A Possible Basis for Mediating Cell Differentiation

The picture that emerges from these studies (Chart 6) is that a cell passing through S phase must initially relax the chromatin structure and open up the segments that are about to be replicated. After initiation the DNA replication proceeds in these segments by a discontinuous process involving the synthesis of short DNA segments that are subsequently ligated together. The success of the DNA replication process depends on an adequate supply of cytoplasmic proteins; these proteins in turn become associated with the newly replicated DNA to form a protein-rich immature form of chromatin. In the living cell the initial

protein-rich associations are then subject to maturation; during this process some of the proteins appear to be extruded from the structure, thereby giving the chromatin a heavier density when analyzed in a CsCl density gradient. It appears highly probable that the introduction of new histones plays an important role in driving this process.

With respect to the possible role of chromatin replication in cell differentiation, it is proposed that the spectrum of cytoplasmic proteins that are available for the initial complexing of the newly replicated DNA can ultimately determine the character of the matured chromatin complexes and thus determine whether the DNA of such complexes will be in a genetically expressible state. It is proposed that, as the spectrum of these proteins is either shifted in amounts, availability, or activity by prior nutritional, hormonal, or physical treatment of the cells, the probability of forming certain chromatin complexes is also changed and that such changes will determine the range of genetically active chromatin complexes formed or stabilized during nuclear replication. Although our knowledge of these processes is still very elementary, it seems probable that with greater insights into the mechanisms of chromatin replication we may be able to direct cell differentiation in ways that will compensate for genetic defects that predispose a cell to malignant growth.

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