Temporal relationships of chromatin protein synthesis, DNA synthesis, and assembly of deoxyribonucleoprotein

(HeLa cell replication/chromatin assembly/chromatin protein redistribution)

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ABSTRACT Chromatin assembly has been investigated in terms of the sites on DNA where newly synthesized chromatin proteins associate. Chromatin from cells labeled with $^{14}$C-BrdUrd and $^{3}$Hlysine was fixed with formaldehyde and resolved in CsCl gradients. By varying the spacing of the labeling intervals of the two isotopes so as to encompass all possible periods in S-phase, the association of labeled, newly synthesized proteins on newly synthesized (BrdUrd-substituted) or preexisting chromatin DNA was determined. In all experiments it was found that newly synthesized chromatin proteins predominately associated with nonreplicating DNA. Possible mechanisms by which cells recycle preexisting chromatin proteins to restore the protein content of newly synthesized DNA are discussed.

During the period of DNA synthesis of the cell cycle (S-phase) the biosynthesis of histones occurs in parallel with that of DNA (1–5). Inhibition of DNA synthesis causes a marked decrease in histone synthesis; this decline in the synthesis of a specific class of proteins is accompanied by the disappearance of histone mRNA (3–10). Conversely, a block in total protein synthesis causes the rate of DNA synthesis to decrease immediately to a residual level, which is maintained for about 1 hr (11–13). The DNA produced in the absence of protein synthesis is deficient in chromatin protein by 50% (12, 13). An analogous finding is that the nascent chromatin DNA of control cells is also transiently deficient in protein by 50% (14). Thus, there appears to be a partially reciprocal mechanism linking DNA synthesis and protein synthesis; the dependence of DNA synthesis on protein synthesis is not understood. Whether continued DNA synthesis requires concurrent synthesis of replication enzymes, chromatin proteins, or as yet undiscovered replication factors, remains to be delineated.

The micrococcal nuclease-resistant DNA synthesized during the block in protein synthesis, and the nuclease-resistant, nascent DNA of replication forks are present in oligonucleotides of sizes characteristic of DNA associated with histones (13, 14). During replication, the addition of histones to newly synthesized DNA follows soon after synthesis of DNA, and is complete within 15 min (14). Thus, there was substantial reason to suspect that newly synthesized histones, and perhaps other chromosomal proteins, associate with newly synthesized DNA in the fork region after replication. Protein would accumulate on regions of protein-deficient DNA produced as a result of doubling of the DNA. Since the bulk of histones and nonhistone chromatin proteins is conserved in HeLa cells during cell division and growth (15), it was of interest to investigate the mechanism by which these proteins are distributed to daughter chromosomes. Recent evidence indicates that the distribution of histones to daughter chromosomes is by a random process (16), in contrast to evidence for nonrandom dispersal of histones to daughter chromosomes (12, 17).

In this communication, the associations of newly synthesized chromatin proteins with DNA have been examined. The conclusion derived from these experiments is that newly synthesized chromatin proteins do not preferentially associate with newly synthesized DNA. Possible mechanisms to account for this observation are discussed.

MATERIALS AND METHODS

HeLa S-3 cells were maintained in Joklik’s modified, Eagle’s minimal essential medium supplemented with 5% fetal calf serum, 5% calf serum, and Tylocin (GIBCO). Cells were checked at biweekly intervals for mycoplasma contamination by the method of Schneider et al. (18). Cultures used for experiments had uridine/uracil incorporation ratios of 300 or greater.

Randomly growing cultures were synchronized with hydroxyurea in two successive cell cycles to obtain cultures in total S-phase synchrony (15). After the cells were released into S-phase for the second time by removal of hydroxyurea, they were suspended either in fresh medium or in labeling medium. Cells were labeled with $^{3}$Hlysine by suspension in medium containing 20% of the normal content of lysine plus 2 $\mu$Ci/ml of L-$^{3}$Hlysine (New England Nuclear, 38.3 Ci/mmol). For density labeling of DNA with BrdUrd, 5-fluorodeoxyuridine (FdUrd) was added to $10^{-5}$ M, BrdUrd was added to $3 \times 10^{-6}$ M, and $^{14}$C-BrdUrd was added to 0.05 $\mu$Ci/ml (Schwarz/ Mann, 56.9 mCi/mmol). Labeling was terminated effectively for both lysine and BrdUrd by washing the cells twice in fresh medium followed by resuspension in fresh, warm medium. Incorporation of both isotopes began after a brief lag of about 15 min and proceeded at a linear rate to the termination point dictated by experimental protocol (tetz).

Cells were harvested by centrifugation and washed twice in 2 mM sodium phosphate (pH 7.2), 0.1 M sucrose. The pellet was frozen at −70°C and stored at −20°C. Further procedures were at 4°C. Chromatin was prepared according to the method of Hancock (19). Cells were suspended in 0.5% Nonidet P-40 (Shell), 2 mM EDTA, and gently homogenized by four strokes in a Dounce homogenizer. Chromatin bodies were collected by centrifugation at 4000 rpm for 10 min in the Sorvall SS-34 rotor; the homogenization process was repeated twice and chromatin was pelleted first at 6000 rpm and then at 8000 rpm. The chromatin pellet was resuspended twice in 0.2 mM EDTA (pH 7.2) and collected by centrifugation at 10,000 rpm for 10 min. The final pellet was suspended in 5 ml of 0.2 mM EDTA and dialyzed against 4 liters of $10^{-5}$ M triethanolamine (pH 7.2). Chromatin in 10 mls of $10^{-5}$ M triethanolamine was sheared in a Virtis 45 homogenizer at 100 V for 2 min. Sheared chromatin was centrifuged for 10 min at 10,000 rpm, and the supernatant was fixed with 1% formaldehyde (titrated to pH 7.5 with NaOH) according to Jackson and Chalkley (20). After fixation for 1 hr, the chromatin was dialyzed against 4 liters of $10^{-5}$ M triethanolamine at 4°C. Fixed chromatin was then di-
The various chromatin proteins are digested with 1 μg of proteinase K (EM Laboratories) per μg of chromatin DNA at room temperature for either 15 or 30 min with equivalent results. Solid guanidine-HCl (1.6 g) (Schwarz/Mann, ultra-pure) was then added and dissolved, and the temperature was returned to 4°C. CsCl (2.0 g) was added, the volume was adjusted to 5.5 ml, and centrifugation was performed at 33,000 rpm, 4°C for 48 hr in a Spinco SW50.1 rotor or in a Spinco SW41 rotor (tubes were brought to volume with paraffin oil).

Gradients were fractionated by collecting drops from the bottom of the tube. Fifty micrograms each of serum albumin and DNA were added to each fraction, followed by addition of 2 ml of 10% trichloroacetic acid. Precipitates were collected on Reeve Angel 1034 AH filters, rinsed twice with trichloroacetic acid, and twice with 95% ethanol. Filters were prepared for assay of radioactivity by digestion with 0.1 ml of NCS (Amersham/Searle) water (9:1), and assayed in 2,5-diphenyloxazole–1,4-bis[2(5-phenyloxazolyl)]benzene (PPO–POPOP) (Liquifluor, New England Nuclear) in a Beckman LS-100 scintillation counter. ¹⁴C spill was corrected for by the automatic external standard method.

RESULTS

The separation attainable between BrdUrd-substituted chromatin and chromatin of normal density in buoyant density gradients is small (Fig. 1A, refs. 19 and 38). This lack of resolution is attributable to chromatins that mask the density differences of the respective DNA species. To better resolve the BrdUrd-substituted chromatin from native chromatin, I used partial proteolysis to remove part of the protein (range 40–60% in various experiments), leaving covalently attached radioactive peptides. The assumption underlying this procedure is that

![FIG. 1. Separation of BrdUrd-substituted chromatin from chromatin of normal density in guanidine-HCl-CsCl gradients. Cells labeled for one generation with [¹⁴C]BrdUrd were mixed with cells labeled for one generation with [¹⁴C]thymidine. After preparation and fixation, chromatin was applied directly to the gradients (A), or was digested with proteinase K (B) prior to centrifugation (Materials and Methods). Density increases from right to left. (O) [¹⁴C]; (●) [³H].](image)

histone and nonhistone chromatin proteins are digested to approximately equivalent degrees. After proteolysis, the BrdUrd-labeled chromatin and control chromatin could be resolved as partially overlapping peaks (Fig. 1B).

Reconstitution experiments were performed to determine the amount of protein exchange during chromatin preparation. Cells that had been incubated with [¹⁴C]BrdUrd for 24 hr were mixed with cells labeled for 24 hr with [³H]lysine. Chromatin was prepared, fixed with formaldehyde, digested, and centrifuged to equilibrium in guanidine-HCl-CsCl gradients (Materials and Methods). The result was equivalent to that shown in Fig. 1B: no transfer of [³H]-labeled protein to the [¹⁴C]-BrdUrd-labeled DNA was observed. This same result was obtained by using cells labeled for 4 hr with BrdUrd, followed by 4 hr of incubation with [³H]lysine. Thus, in agreement with others (19, 20), protein exchange during this procedure was found to be minimal.

In early experiments performed to study the time-frame of association of newly synthesized proteins with newly synthesized DNA, no such associations could be demonstrated after repeated attempts. The following is an experimental approach designed to determine unambiguously whether these proteins did not, in fact, associate with newly replicated DNA.

To examine the association of newly synthesized proteins with respect to newly replicated DNA, I synchronized cells in S-phase by two successive treatments with hydroxyurea (15). During the second S-phase, in which the population proceeded through S-phase synchronously, the cells were incubated for a specified period with the density precursor, [¹⁴C]BrdUrd, and for specified periods in S-phase with [³H]lysine to label chromatin proteins. The labeling protocols are diagrammed in Fig. 2.

![FIG. 2. Labeling protocols for density-labeling chromatins DNA and radiolabeling chromatins proteins.](image)

![FIG. 3. Association of newly synthesized proteins with DNA. Cells were labeled according to experiment 1 in Fig. 2. [¹⁴C]BrdUrd incorporation was during the first 3 hr of S-phase; [³H]lysine incorporation was during 0-3 hr (A), 3-6 hr (B), and 6-9 hr (C) of S-phase. Density increases from right to left. (O) [¹⁴C]; (●) [³H].](image)

![FIG. 4. Association of newly synthesized proteins with DNA. Cells were labeled according to experiment 2 in Fig. 2. [¹⁴C]BrdUrd incorporation was during the middle third of S-phase; [³H]lysine incorporation was during 0-3 hr (A), 3-6 hr (B), and 6-9 hr (C) of S-phase. Density increases from right to left. (O) [¹⁴C]; (●) [³H].](image)
2. In experiment 1, for example, cells were incubated with \(^{14}\text{C}\)BrdUrd during the first one-third of the S-phase (hr 0–3). The culture was divided into three aliquots for protein labeling: one was incubated simultaneously with \(^{3}\text{H}\)lysine and \(^{14}\text{C}\)BrdUrd during the initial 3 hr of S-phase, one aliquot was incubated with \(^{3}\text{H}\)lysine during the middle one-third of S-phase (hr 3–6), and the third aliquot was labeled with lysine during the final 3 hr of S-phase (hr 6–9). Chromatin was prepared from the three samples, fixed with formaldehyde, digested with proteinase K, and centrifuged to equilibrium in isopycnic gradients (Materials and Methods). In experiments 2 and 3, BrdUrd incorporation was performed at different sections of S-phase; otherwise, the experiments were identical. It was anticipated that if newly synthesized proteins associated with newly synthesized DNA, then, when density and amino acid labeling were performed simultaneously, the radioactivity peaks would coincide. Likewise, when labeling was noncoincident, the peaks should be noncoincident.

Experiment 1 of the labeling protocols depicted in Fig. 2 is shown in Fig. 3. Cells were labeled in the first one-third of S-phase with \(^{14}\text{C}\)BrdUrd, and with \(^{3}\text{H}\)lysine during the first, middle, and last one-third of S-phase. In all three cases, the \(^{3}\text{H}\)-labeled peptides were associated with DNA of light density, not with the DNA of increased density. The position of the tritium peak is the position of chromatin of normal density, prepared in the same manner and run as a control in each experiment. In addition, when protein labeling was performed simultaneously with BrdUrd incorporation, as in Fig. 3A, and the cells were then allowed to proceed through S-phase before cell harvest, the radioactive protein was also associated with DNA of light density.

Experiments 2 and 3 of Fig. 2 were performed to further investigate the nonassociation of newly synthesized chromatin proteins with newly synthesized DNA. Experiment 2 is perhaps the most lucid of the three, because protein labeling is performed before, simultaneously with, and after the period of \(^{14}\text{C}\)BrdUrd incorporation (Fig. 4). The results were the same. In no case was newly synthesized protein found associated with density-substituted DNA.

In experiment 3, BrdUrd incorporation was during the final 3 hr of S-phase, while \(^{3}\text{H}\)lysine incorporation was performed in each third of S-phase. The result (Fig. 5) was in accord with those of Figs. 3 and 4; i.e., no preferential association of newly synthesized protein with newly synthesized DNA was detected.

In Fig. 4, and to a lesser extent in Figs. 3 and 6, a peak of light density containing tritium, but no \(^{14}\text{C}\), is seen. This is due to incomplete proteolytic digestion, in which crosslinked peptides are cleaved from DNA and are not hydrolyzed to acid-soluble material by the termination of the digestion step. In these gradients, containing 3 M guanidine-HCl, the buoyant density of protein is such that proteins enter the gradient, whereas in standard CsCl gradients, the proteins float at the meniscus.

Several artifacts that could produce the observed results were considered. If the periods of incubation with both isotopes were not representative of the true incorporation periods due to, e.g., large lysine pools that delayed \(^{3}\text{H}\)lysine incorporation for a long period, and \(^{14}\text{C}\)BrdUrd incorporation was complete by the time significant lysine incorporation had begun, then incorporation of the two isotopes would actually be nonsimultaneous. In fact, however, control experiments demonstrated that incorporation of each isotope was linear for the duration of the experiment.

A second control experiment was performed to demonstrate that the observed results were not caused by the synchrony procedure. Randomly growing cells were incubated simultaneously with \(^{14}\text{C}\)BrdUrd and \(^{3}\text{H}\)lysine, and samples were removed for chromatin preparation after 6, 12, 21, and 30 hr (Fig. 6). In the earliest sample, the density-substituted DNA bands separately from the light DNA bearing \(^{3}\text{H}\)lysine-containing peptides, in accord with the above results. The \(^{3}\text{H}\)-protein band shifts to the density-substituted DNA region progressively, as the DNA is converted to the heavy-light hybrid. DNA synthesis in the presence of BrdUrd is slower than...
in control cells (21), and thus the generation time of HeLa cells growing under these conditions is somewhat prolonged.

Chromatin containing one DNA strand substituted with BrdUrd has a normal protein content, and the proteins do not appear to bind the DNA with significantly altered affinities (21). If proteins did not associate with the newly made, BrdUrd-substituted DNA, thus leaving a protein deficiency on the density-substituted chromatin DNA, this chromatin would have a markedly heavier density than that observed. Conversely, if preexisting protein binds BrdUrd-substituted DNA so as to interfere with the binding of newly synthesized protein, then label would not accumulate at the sites of DNA synthesis. In experiments to be detailed elsewhere, cells labeled with [3H]lysine in specified portions of one S-phase were incubated with BrdUrd in the corresponding segments of the succeeding S-phase. In agreement with the results presented above, protein and DNA synthesized in the same S-phase period of two consecutive cell cycles were not found to be associated.

These studies indicate that, during chromatin biosynthesis, as DNA is replicated, the protein-binding sites created as a result of doubling of the DNA content are filled with recycled chromatin proteins, and not with newly synthesized chromatin proteins.

**DISCUSSION**

In this communication, experiments are described in which the addition of proteins to DNA during chromatin replication is examined. It is apparent that preexisting protein is utilized for the replenishment of protein in the fork region after replication (14).

It is not presently known whether chromatin proteins are displaced during replication (and transcription) as the enzymes proceed along with template DNA, or whether chromatin proteins interfere at all with replication or transcription machinery; i.e., they may remain bound during nucleic acid synthesis. These results, together with those in the accompanying article (22), indicate that chromatin protein redistribution must occur during DNA replication to account for the accumulation of recycled proteins on newly synthesized DNA soon after DNA synthesis (14).

One can envisage that redistribution is accomplished either by protein sliding or by dissociation and reassociation of the proteins. The sliding mechanism must reconcile tandem replication regions (11, 29–26) in which replication forks of neighboring replicons proceed toward one another. Alternatively, proteins may dissociate and reassociate. Two mechanisms could account for this. In one, the replication, and possibly transcription, machinery may displace proteins during their passage along the DNA. Such a process would create regions of essentially naked DNA behind the replication fork or the RNA polymerase. In the case of DNA synthesis, however, the newly replicated region is not naked, but has half the protein content of mature chromatin (12–14) and the DNA associated with proteins is associated with histones (13, 14). By a second mechanism, histones are not displaced as a result of replication, but histone proteins may dissociate spontaneously from all chromatin sites and reassociate randomly with DNA, and in this manner fill the "vacancies" on the replicated chromatin DNA. Either mechanism for redistribution will create a nonbound chromatin protein pool. Evidence for the existence of these pools has been reported (13, 27). A labeled, newly synthesized protein entering the nucleus would then compete with this pool of unlabeled proteins for available sites on DNA. This simple isotope dilution effect could explain the observation that newly synthesized proteins are not found associated with the new DNA.

It is an interesting observation that during prolonged inhibition of protein synthesis, the DNA generated has half the histone content of mature chromatin (12–14). If dissociation of histones were purely spontaneous in vitro, the DNA replicated during a 1 hr period of cycloheximide inhibition should have much of its protein content restored. Thus, it appears that the mechanism for association of proteins with DNA in the region of the replication fork is one that requires concomitant protein synthesis. The nature of this requirement may be a key to understanding the depression of DNA synthesis after inhibition of protein synthesis (12–14, 28) and the very low rate of DNA synthesis attainable in vitro (29, 30), where the DNA synthesis and protein synthesis systems have been physically uncoupled.

The observations described herein may relate to certain characteristics of simian virus 40 and polyoma virus infections. These viruses stimulate cellular replication upon infection; a primary function of this stimulation may be to provide a chromosomal protein supply (51–23). Cellular histones labeled prior to infection, as well as histones labeled after infection, are found associated with viral DNA (34, 32). Viral DNA synthesized in the presence of a protein synthesis inhibitor is deficient in protein (35–37), in analogy to the situation in uninfected cells in which protein synthesis is blocked (12, 13). Thus, it appears that in viral replication also, the protein redistribution mechanism is dependent upon concomitant protein synthesis.

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