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Deposition of histone onto the replicating chromosome: Newly synthesized histone is not found near the replication fork

(cell replication/histone synthesis/DNA synthesis/chromatin assembly)

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ABSTRACT **We have studied the site of deposition of newly synthesized histone. It appears to be randomly distributed over the chromosomal material and does not become associated specifically with immediately post-replicational DNA, nor is it deposited in discrete continuous regions distal to the sites of DNA synthesis. The newly synthesized DNA, however, rapidly acquires a complement of chromosomal proteins; presumably, preexisting histones must migrate to become associated with post-replicational DNA.**

Histones are synthesized in the cytoplasm (1, 2) of dividing cells in S phase (3, 4). They are subsequently transferred into the nucleus with great rapidity (5–8). Both preexisting and new histones become associated with the doubled complement of chromosome DNA in a random manner (9), so that previous interactions with specific strands or DNA molecules are not necessarily maintained. *A priori*, it seemed likely that incoming histone would become associated with newly synthesized DNA either just ahead of or just after the replication fork. In view of this likelihood, a number of theories have been proposed for histone F_1 phosphorylation which occurs within a few minutes after admission to the nucleus. These theories deal with changes in histone and chromatin conformation required for replication which are likely to occur in the neighborhood of the replication fork. This seemed quite feasible if DNA replication occurred on the periphery of the nucleus as had been suggested by earlier work (10, 11). However, the clear-cut demonstration that DNA synthesis is fully active within the body of the nucleus posed fascinating problems of how the positively charged, incoming histones could circumnavigate the bulk of the chromosomal material on its passage to an inner replication fork (12, 13).

We have asked specific questions concerning the site of deposition of incoming histones and we will argue that newly synthesized histones do not in fact become associated specifically with DNA in the region of the replication forks, but rather become randomly associated with the bulk chromatin. We further conclude that the newly synthesized DNA assumes its full complement of histone from preexisting molecules which presumably migrate from other chromosomal sites. This migration is possibly triggered by the incoming flux of histone into the nucleus.

MATERIALS AND METHODS

Labeling of Rat Hepatoma Tissue Culture Cells, Isolation of Chromatin, and Analysis on CsCl Gradient. Four hundred milliliters of rat hepatoma tissue culture (HTC) cells in mid-log phase (4×10^5 cells per ml) were labeled with [^{3}H]lysine (500 μCi, New England Nuclear) for 30 min. The cells were harvested and resuspended in fresh medium (Swims S-78) in the presence or absence of 5′-iododeoxyuridine (IdUrd) at 0.1 μM. For the 30-sec pulse experiments, the cells were concentrated to a cell density of 4×10^7 cells per ml (20 ml total volume) prior to incubation with 2.0 mCi of either [^{3}H]lysine or [^{3}H]thymidine. The incubation was stopped by addition to 180 ml of cold medium; the cells were washed once and then resuspended in warm medium at a cell density of 4×10^5 cells per ml for the chase. After the cells had been harvested, they were washed once in an equal volume of 0.1 M sucrose, 0.2 mM Na_2HPO_4, pH 7.4, at 4° and quickly frozen in dry ice–ethanol. Identical results were observed if the cells were not frozen prior to isolation of chromatin, which indicates that the freezing did not cause the randomization. Preparation of chromatin was by the procedure of Hancock (14), which involves an initial homogenization in 0.1 M sucrose, 0.2 mM Na_2HPO_4, at pH 7.4, then several washes in 0.5% Nonidet P-40, 0.2 mM EDTA, pH 8.0, two washes in 0.2 mM EDTA to remove the detergent, and a final wash with distilled water to form the chromatin gel. The gel was then vigorously sheared for 1 min at 4° in a Virtis model "45" homogenizer at maximum shear force, and centrifuged at 27,000 × *g* for 20 min to remove membrane fragments. The supernatant which contains the solubilized chromatin was dialyzed against 10 μM triethanolamine, pH 7.4, overnight at 4°, and fixed in 1% formaldehyde for 4 hr at 4°; the excess formaldehyde was removed by dialysis overnight. The chromatin was mixed with guanidine hydrochloride (2.10 g) (Heico, Inc.) and CsCl (1.50 g), and adjusted to a final volume of 5.5 ml in 0.1 M Tris·HCl, pH 8.0. After centrifugation at 35,000 rpm in a Beckman SW 50.1 rotor for 72 hr at 4°, 20-drop fractions were collected and counted in Bray's solution (15) using a Unilux II scintillation counter. The [^{3}H]lysine/[^{14}C]thymidine ratio was kept constant in all gradients to ensure a comparable double label analysis in all concentrations of nucleoprotein over the range 25–500 μg of DNA.

RESULTS

It is most critical, for the analysis to be described, that we can identify the position in a density gradient of labeled histone fixed to DNA which is either of normal density or contains one strand uniformly density-labeled with IdUrd. Such an analysis requires crosslinking of histone to DNA without interstrand crosslinks (16), an assessment of the position of the chromosomal nonhistone proteins in the density gradient (9), and an assurance that histones do not migrate during the isolation of the chromatin (16). An approach to the solution of these problems has been reported previously (9, 16, 17).

The data of Fig. 1A indicate that if the proteins of chromatin are labeled *in vivo* with [^{3}H]lysine and the fixed chromatin examined on CsCl density gradients, then the ^{3}H]lysine is distributed asymmetrically about the light side of the normal

Abbreviations: HTC, rat hepatoma tissue culture; IdUrd, 5′-iododeoxyuridine.

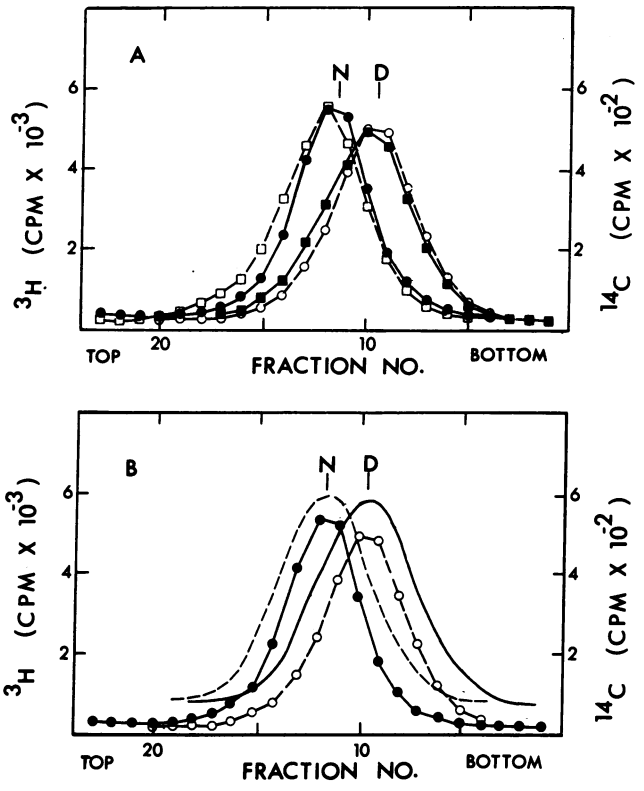

FIG. 1. The distribution of [^{3}H]histone in fixed chromatin. (A) The distribution of [^{3}H]lysine. HTC cells grown for one cell cycle (16 hr) in the presence of [^{14}C]thymidine (-●-●-●-), in the presence of [^{3}H]lysine (-□-□-□-), in the presence of [^{3}H]thymidine and IdUrd (0.1 nM) (-○-○-○-), and in the presence of [^{3}H]lysine and IdUrd (-■-■-■-). (B) [^{3}H]Lysine in histone after correction for nonhistone content in the fixed chromatin of Fig. 1A. [^{3}H]Histone distribution in the absence of IdUrd (- - -) and in the presence of IdUrd (—). The [^{14}C]thymidine and [^{3}H]thymidine-IdUrd distributions are the same as in Fig. 1A. N refers to the position of chromatin containing no density label. D refers to the position of chromatin containing IdUrd.

density distribution of chromatin. When the cells are grown for one cell cycle in the presence of both [^{3}H]lysine and the density label, IdUrd, this asymmetry towards a relatively lower density is maintained, as can be seen when this distribution is compared to fixed chromatin isolated from cells labeled with [^{3}H]thymidine and IdUrd for one cell cycle. This asymmetry is caused by the presence of nonhistone proteins in the fixed chromatin and can be corrected, such that the density distribution of [^{3}H]lysine in histone alone can be determined (9, 17). The correction is based on defining the distribution of nonhistone protein as reflected in the position of [^{3}H]tryptophan in these gradients. One then measures the fraction of [^{3}H]lysine present as nonhistone protein. Then, knowing the distribution of nonhistone protein and the relative amount of histone and nonhistone protein, one can subtract the nonhistone contribution from the total [^{3}H]lysine present in the density gradient. As discussed previously (9, 17), such a correction is based upon the assumption that the distribution of tryptophan and lysine is statistically comparable in the nonhistone protein in the density gradient. Such a correction of the data of Fig. 1A is shown in Fig. 1B. The histone distribution is found to be symmetrical with respect to the distribution of radiolabeled thymidine in the chromatin which is consistent with the feasibility of the correction. Also, Fig. 1B demonstrates that the separation between histone associated with normal or dense-labeled DNA is substantial and sufficient to define whether the histone is

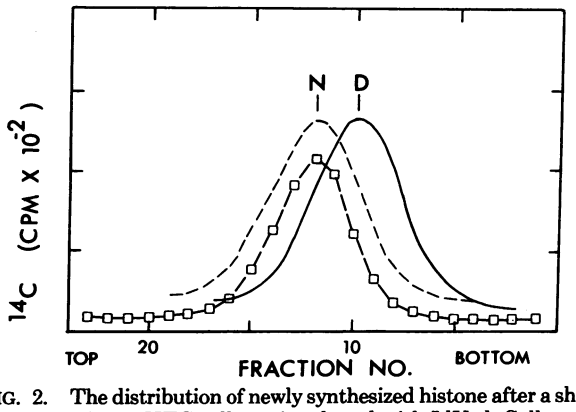

FIG. 2. The distribution of newly synthesized histone after a short [^{3}H]lysine pulse in HTC cells preincubated with IdUrd. Cells were preincubated with IdUrd for 1 hr (- - -) or for 16 hr (—), and [^{3}H]-lysine was added for the final 30 min of the IdUrd pulse. After chromatin isolation and fixation, samples were aligned within the CsCl gradients with respect to a fixed chromatin sample of normal density containing [^{14}C]thymidine (-□-□-□-). A parallel experiment was also performed with [^{3}H]tryptophan so that the correction for nonhistone protein content could be made.

associated with either form of DNA. A more detailed analysis of the correction for the distribution of nonhistone protein is described in a recent paper (9).

The following experiment was performed to test whether newly synthesized histones were deposited in the region of the replication fork. HTC cells were grown for 1 hr in the presence of IdUrd. [^{3}H]lysine was then added for an additional 30 min in the continued presence of IdUrd. Clearly, if the histone is deposited within 1 hr of the passage of the replication fork the [^{3}H]histone will be associated with dense nucleoprotein. The same is true if the histones were deposited prereplicatively within 20–30 min of the passage of the fork.

The results of such an experiment are shown in Fig. 2, and provide an unequivocal demonstration that the newly synthesized, [^{3}H]histones do not become significantly associated with newly synthesized, density-labeled DNA within 1 hr after their synthesis and transport to the nucleus. After a pulse of 16 hr (one full cell cycle) of IdUrd followed by a 30-min [^{3}H]lysine pulse, the [^{3}H]histone is now found associated with IdUrd-containing DNA, which indicates that deposition occurs relatively soon after synthesis and that failure to deposit at the replication fork is not a reflection of failure to deposit elsewhere. Similar experiments with 3-hr pulses of [^{3}H]lysine lead to similar observations and, thus, exclude the likelihood that the histones are residing in a nuclear pool, as no indication of so massive a pool has been reported.

We can conceive of several possible interpretations of these data: (*A*) histone is associated with the newly replicating DNA for a short time interval, but subsequently randomizes; (*B*) histones are desposited at a discrete, continuous region of the chromosome at a defined (and considerable) distance behind the replication fork; (*C*) histone is deposited well ahead of the replication fork; or (*D*) histones are, in effect, randomly deposited on the chromatin.

The data in Fig. 3 confirm the earlier observations of Fakan *et al.* (13) and of Seale and Simpson (18) that, immediately (30 sec) after synthesis, newly synthesized DNA is associated with chromosomal material of unusually low density, but that after 30 min it is associated with normal density chromatin. After similar times of exposure to [^{3}H]lysine, the [^{3}H]histone is associated only with normal density chromatin and does not show any shift from an unusually light to normal density chromatin expected if it were deposited at the replication fork. This ex-

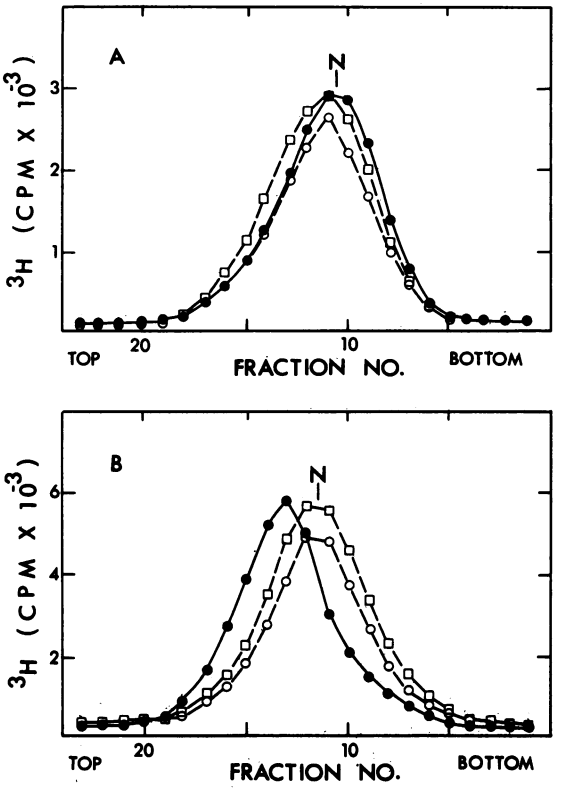

FIG. 3. The [^{3}H]lysine and [^{3}H]thymidine distribution of fixed chromatin after a 30-sec pulse and the subsequent chases. (A) [^{3}H]-Lysine distribution after the 30-sec pulse (-●-●-●-), after a 30-min chase (-□-□-□-), and after a 60-min chase (-O-O-O-). (B) The [^{3}H]thymidine distribution after the 30-sec pulse (-●-●-●-), after a 30-min chase (-□-□-□-), and after a 60-min chase (-O-O-O-). All samples were aligned with respect to a fixed chromatin sample containing [^{14}C]thymidine (data not shown). The [^{3}H]lysine distribution reflects primarily the histone distribution in these gradients. The reason for this is the small amount of [^{3}H]lysine taken up into nonhistone protein in a 30-sec pulse. Therefore, the [^{3}H]tryptophan correction was not applied.

periment also indicates that certainly after 30 min, newly synthesized DNA must be associated with a normal density complement of chromosomal protein, and, because this does not include newly synthesized histone, it must *a priori* consist of either old preexisting histone molecules or a superabundance of nonhistone protein. This experiment was repeated in the presence of IdUrd; no deposition of the newly synthesized histone on newly synthesized IdUrd DNA either after the brief pulse or during the chase period was seen (data not shown).

A final series of experiments were performed to test if the histones become randomly distributed over the chromosomal material or whether histones become associated with a discrete, continuous region of the chromosome far removed from a replication fork. HTC cells were labeled with [^{3}H]lysine for 30 min. The labeled amino acid was removed and IdUrd was then added. Samples were collected at 5-hr intervals up to 15 hr, and analyzed for histone distribution. The results are shown in Fig. 4. There is a gradual, but steady shift of the [^{3}H]histone onto dense DNA during the course of the experiment. Certainly, there is no sudden shift which would be anticipated if the incoming histones were to be associated with a short continuous region of the chromosome.

In a second experiment, the [^{3}H]lysine was incorporated into histone in a 30-min pulse and in a series of separate flasks, IdUrd was added at 0, 3, 6, 9, or 12 hr after the [^{3}H]lysine pulse. The

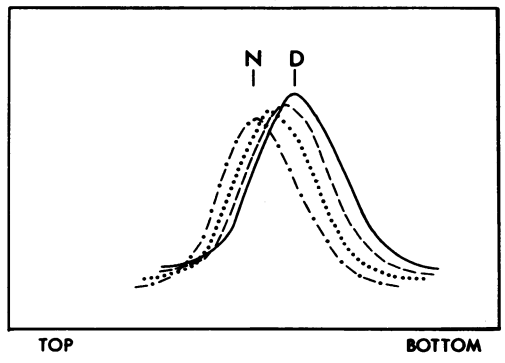

FIG. 4. The newly synthesized histone distribution after a 30-min [^{3}H]lysine pulse and 1-hr chase followed by IdUrd for increasing periods of time. The histone distribution immediately after the pulse and 1-hr chase (·—·), after 5 hr in IdUrd (·····), after 10 hr in IdUrd (– – –), and after 15 hr in IdUrd (—). All samples were aligned with respect to a fixed chromatin sample containing [^{14}C]thymidine (data not shown). The experiment was also done with [^{3}H]tryptophan to correct for nonhistone protein content.

IdUrd was incorporated into the DNA for 3 hr at each time point. In no instance was there a sudden shift of [^{3}H]histone into dense chromatin, which further indicates that the newly synthesized histones do not become associated with the chromosomal material in a discrete continuous manner along the DNA (data not shown). This experiment also excludes the possibility that immediately pre-replicative deposition occurs. We conclude, therefore, that histones are deposited randomly over the chromosome and only become totally associated with denser DNA when the full complement of DNA has been so labeled.

DISCUSSION

The experiments described in this paper indicate that newly synthesized histones are not deposited in a discrete, orderly manner near to the replication fork, neither post-replicatively nor immediately pre-replicatively. This is consistent with the observation reported by Seale (19) in the accompanying paper. Further, the experiments described in Fig. 4 indicate that the incoming histone must be randomly dispersed over the chromosome, and that it did not become associated with the chromosome in discrete, continuous units, albeit removed from the replication fork.

In view of the overall negative charge of the chromosomal material and the polycationic nature of histones, these results are not surprising. Indeed, it would have been remarkable if incoming histones could have found their way through the surrounding chromatin specifically to the replication forks. However, although it is clear that incoming histones do not become associated with newly synthesized DNA, nonetheless, the immediately post-replicated chromosomal material is not abnormally dense, which would indicate a less-than-normal complement of histones. We conclude that either the postreplicational DNA is associated with an unusually large amount of nonhistone protein, or we must adopt the conclusion that preexisting histones must migrate to the newly synthesized DNA in order to maintain the normal histone:DNA ratio. Possibly the incoming, newly synthesized histone might act as a trigger for such histone migration. It has previously been shown *in vitro* that histone exchange can take place between different nucleoprotein strands if 0.15 M NaCl or divalent cations are present in the system (9, 17).

The biological import of these observations is difficult to assess because we do not have sufficient information about the

details of the process, for example, whether a specific histone is replaced by an identical molecule or by any other histone.

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