Proc. Nat. Acad. Sci. USA
Vol. 72, No. 11, pp. 4440–4444, November 1975
Cell Biology

Deposition of histones onto replicating chromosomes

(chromosome replication)

VAUGHN JACKSON, DARYL K. GRANNER, AND ROGER CHALKLEY

Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242

Communicated by F. H. C. Crick, August 6, 1975

ABSTRACT **The mode of distribution of newly synthesized and pre-existing histones has been studied during the process of chromosome replication. Newly synthesized histone was labeled with [^{3}H]lysine and newly synthesized DNA was density labeled with iododeoxyuridine. The histone was covalently linked to DNA, and radiolabeled histone was analyzed on CsCl density gradients. We have defined conditions that do not give rise to histone randomization during isolation, and also developed a method of defining the distribution of histones in chromatin on a density gradient in the unavoidable presence of nonhistone protein.**

Three possible modes of distribution of histone onto the replicating chromosome can be conceived; we describe experiments designed to distinguish unequivocally among these possibilities and conclude that histones are deposited randomly onto the chromosome.

Three models for histone distribution are possible: (*a*) conservative, in which all the old histone is associated with only one daughter DNA molecule; (*b*) semiconservative, in which old histones retain an interaction with one specific strand in each daughter DNA molecule; and (*c*) a fully random mode. Autoradiographic studies by Prescott and Bender (1) after a pulse of labeled amino acids in amoebae indicated that in subsequent generations the radiolabel is uniformly associated with all chromatids. Unfortunately, this approach cannot distinguish between histones and nonhistone proteins. The results of Prescott and Bender are entirely consistent with a random mode of histone deposition, but of course they do not prove this point. More recently, Tsanev and Russev (2) have argued that at replication, old histones remain associated with the old DNA strands and that newly synthesized histone is associated with the new DNA strand.

We felt, therefore, that it would be appropriate to adopt a different approach utilizing incorporation of density label into DNA (3, 4) and analyzing fixed (5, 6) chromatin preparations in CsCl density gradients. Our results indicate that histones are distributed on the replicating DNA in a random manner.

MATERIALS AND METHODS

Experimental procedures for labeling of hepatoma tissue culture cells (HTC), isolation of chromatin, and centrifugation in CsCl gradients are as previously described (4, 10).

RESULTS

We can conceive of three ways whereby newly synthesized histone could deposit on DNA: conservative, semiconservative, and random deposition. The conservative and semiconservative modes of deposition can be subdivided into several different forms (Table 1).

In Fig. 1 we see an example of conservative deposition in which the newly synthesized histone is deposited on the daughter DNA molecule which contains the younger parental DNA strand; conversely the pre-existing histone is distributed so that it is in association with the daughter DNA molecule which contains the older strand from the parent DNA (Table 1, Model 1). Although it is not shown in Fig. 1, there is another mode of conservative deposition in which the newly synthesized histone becomes associated with a DNA molecule containing the older parental strand (Table 1, Model 2). One possible form of the semiconservative mode is shown in Fig. 1. In this example the pre-existing histone at the time of replication distributed itself equally onto

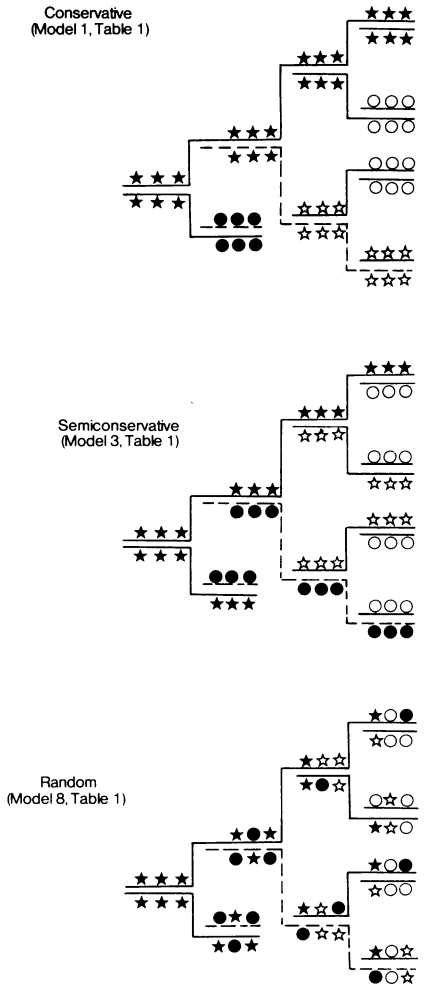

FIG. 1. Typical models for organization of histone during chromosome replication. First generation of histones, ★; second generation, ●; third generation, ☆; fourth generation, ○. Each branch point represents the completion of a full cell generation. The dashed line represents the subsequent distribution of a density label incorporated during the second generation.

Abbreviation: IdUrd, iododeoxyuridine.

Table 1. Distribution schemes for histone deposition

Model	Experiment 1			Experiment 2			Experiment 3			
	Pulse	Chase	Chase	Pulse	Chase	Chase	Pulse	Chase	Chase	Chase
A. Conservative										
1. Old histone on older DNA strand	D,	N,	N	D,	D,	D	N, D,	N,	N	
2. Old histone on newer DNA strand	D,	D,	N	N,	N,	N	N, D,	D,	N	
B. Semiconservative										
3. Old histone on old DNA strand	D,	D,	D	50% D,	N,	N	N, D,	N,	N	
4. Old histone on new DNA strand	D,	N,	N	50% D,	50% D,	N	N, D,	D,	N	
5. Old histone on old DNA strand (but strand switch)	D,	N,	N	50% D,	50% D,	N	N, D,	D,	N	
6. Old histone on new DNA strand (strand switch)	D,	D,	D	50% D,	N,	N	N, D,	N,	N	
7. Old histone on new/old strand alternating	D,	D,	N	50% D,	25% D,	N	D, N,	N,	N	
C. Random										
8. Random with respect to DNA strands and molecules	D, 50% D, 25% D			50% D, 25% D, 12.5% D			N, D, 50% D, 25% D			

D, high density nucleohistone containing one strand labeled with iododeoxyuridine and one strand of normal density DNA; N, normal density nucleohistone.

both daughter DNA molecules and upon subsequent division is always associated with the same DNA strand (Table 1, Model 3). This is the model for histone deposition proposed by Tsanev and Russev (2). We can conceive of five variants of this mode of deposition, depending upon whether incoming histone is associated with a pre-existing or a newly synthesized DNA strand and whether the histones could switch strands at replication. The third model for organization of histones during chromosome replication is the random approach (Table 1, Model 8), and this should be adequately defined in Fig. 1.

We have conducted a series of three types of experiments designed to distinguish among these various possibilities. The strategy involved is discussed below with specific reference to the results one might predict if the semiconservative model (Fig. 1) presented in Table 1 (Model B-3) were to be that most accurately depicting events occurring in the cell.

Exp. 1 involves growing HTC cells in the presence of both [^{3}H]lysine and iododeoxyuridine (IdUrd) for 16 hr (one full cell cycle). Under these conditions all of the newly synthesized DNA strands in the chromatin contain the density label, and the newly synthesized histone contains [^{3}H]lysine. This is the pulse period shown for Exp. 1 in Table 1. Both the IdUrd and [^{3}H]lysine are then replaced by fresh medium containing 10^{-5} M thymidine and chased for 16 hr and 32 hr, respectively. Under these conditions the chase period continues for two full rounds of replication. If the semiconservative mode of deposition (Table 1, Model B-3) were to be correct, we predict that after the pulse of [^{3}H]lysine and IdUrd, the newly synthesized [^{3}H]lysine-labeled histone will be deposited on DNA molecules containing the heavy base and the nucleoprotein will therefore be more dense (D) than normal nucleohistone (N). After the first chase (16 hr) the [^{3}H]lysine-containing histone is still associated with DNA containing the heavy base. Likewise after the second chase of 16 hr the [^{3}H]histone would still be associated only with those chromosomes containing the density-labeled DNA.

The second set of experiments involves incubating cells with IdUrd for 16 hr and then, after removal of the IdUrd, treating the cells with [^{3}H]lysine for 16 hr. This latter incubation is the pulse of Exp. 2 in Table 1. Under these conditions the newly synthesized histone is now deposited on DNA, half the molecules of which contain one dense strand and the other half of the DNA molecules contain both strands of normal density. Thus at the conclusion of the [^{3}H]lysine pulse period the semiconservative model of Table 1 (Model B-3) predicts that radiolabel will be associated with two classes of nucleoprotein, one dense and one of normal density; this is designated 50% D, 50% N in Table 1. After a chase period (no [^{3}H]lysine or IdUrd in the medium) for 16 hr, this model predicts that the [^{3}H]lysine will no longer be associated with dense DNA and the radiolabeled nucleoprotein should have the same density as normal nucleohistone (N). Similarly during a second chase of 16 hr this model predicts that the [^{3}H]lysine-labeled nucleohistone will continue to be of normal density (N, Table 1).

The third set of experiments involves incubating cells with [^{3}H]lysine for 16 hr (pulse period of Exp. 3, Table 1) and after removal of [^{3}H]lysine, treating the cells with IdUrd for 16 hr (first chase of Exp. 3, Table 1). The [^{3}H]lysine after the pulse period should be of normal density (N), and after the first chase it is expected to be dense (D) as it is now associated with DNA molecules, all of which contain the dense base. However, during the second chase of 16 hr initiated immediately after removal of IdUrd, the [^{3}H]histone will be on the DNA strand that does not contain the density label and, therefore, the labeled nucleoprotein will be of normal density (N). One would predict that during a third chase of 16 hr the [^{3}H]lysine will continue to be associated with chromatin that is of normal density (N). Applying similar reasoning to the eight models of Table 1 leads to a series of specific predictions concerning the association of ^{3}H-labeled histones with density-labeled DNA. The predictions documented in Table 1 are sufficiently unambiguous that one can discriminate clearly among the three general models for deposition and to a degree among the variants within each general model.

In order to measure the density changes due to the presence of IdUrd, the nucleohistone isolated from these cells was fixed with formaldehyde and then examined on CsCl

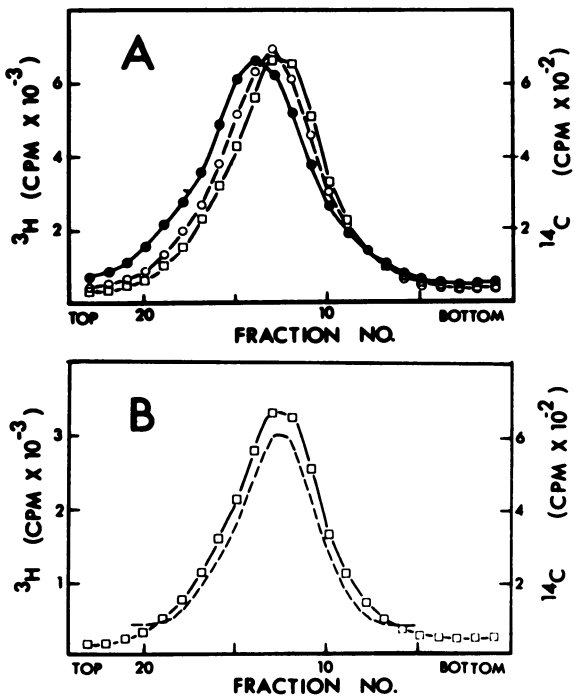

FIG. 2. Determination of distribution of [^{3}H]lysine in histone in the presence of nonhistone protein. (A) HTC cells were grown in the presence of [^{3}H]tryptophan, [^{3}H]lysine, or [^{14}C]thymidine for 16 hr. Chromatin containing [^{14}C]thymidine (□) was mixed with either [^{3}H]lysine-labeled chromatin (○) or the [^{3}H]tryptophan-labeled chromatin (●) and was centrifuged in CsCl. (B) Corrected distribution of [^{3}H]lysine in histone (- - -) relative to [^{14}C]thymidine-containing chromatin (□). The correction is described in the *text* and is critically dependent upon a determination of the amount of [^{3}H]lysine in both histone and nonhistone protein.

density gradients. Previous studies have shown (4, 10) that the density differences are sufficiently large that one can readily distinguish between normal (N) and dense (D) nucleohistone.

The experiments to analyze histone distribution during replication require that we have a knowledge of the distribution of radiolabeled histone in the CsCl gradients of fixed chromatin. Unfortunately, nonhistone proteins not only incorporate [^{3}H]lysine, but also are fixed within the chromosomal complex with some efficiency. It is necessary therefore to apply a correction for distribution of nonhistone protein in order to assay precisely for the position of [^{3}H]histone within the density gradients. This correction can be performed by exploiting the observation that histones do not contain tryptophan, which allows one to use this amino acid as a marker for density distribution of nonhistone protein in CsCl density gradients. This approach is described in detail below and utilizes the results presented in Fig. 2A, which show the distribution of [^{3}H]tryptophan in nonhistone proteins and of [^{3}H]lysine in both nonhistone proteins and in histones. The amount of [^{3}H]lysine that is present in nonhistone proteins is determined directly on all samples before fixation and centrifugation using acid solubility and gel analysis. In general we observed that about 50% of the label is nonhistone protein and about 50% is found in histones. We then compare the density distribution profile of [^{3}H]tryptophan (nonhistone) to that of the [^{3}H]lysine (nonhistone + histone) using a common internal standard ([^{14}C]thymidine in nucleohistone added to each sample). The area under the curve showing the distribution of [^{3}H]tryptophan was adjusted to be a required fraction (i.e., the fraction of the total [^{3}H]lysine counts due to nonhistone protein) of the area of the [^{3}H]lysine density distribution using a Dupont curve analyzer, so that the shape of the curve is maintained faithfully even though the area is changed. The adjusted nonhistone [^{3}H]tryptophan curve is then subtracted from the (nonhistone + histone) [^{3}H]lysine curve to give the histone distribution curve in the CsCl density gradient. The data shown in Fig. 2A were corrected in this way and are shown in Fig. 2B.

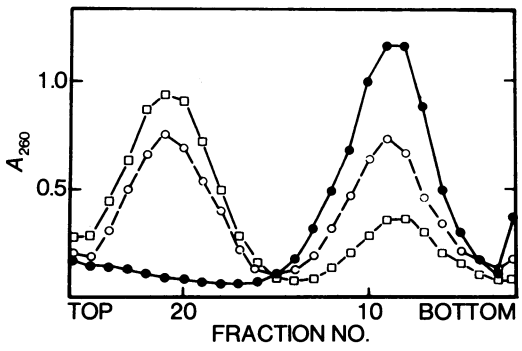

FIG. 3. Precision of measurement of cell generation time. HTC cells were pulsed with IdUrd for 16 hr and an aliquot was collected. The remainder of the cells were washed and permitted to grow in fresh medium for either 16 or 32 hr. Chromatin was isolated and applied to CsCl solutions without fixation so that the distribution of DNA could be measured. DNA in the cells after 16 hr of density pulse (●), after 16 hr of chase in the absence of density label (○), after 32 hr of chase (□).

In order for these experiments to be meaningful it is important to demonstrate that histones are not redistributed among DNA molecules during the preparative procedure. To test for this possibility, we utilized the approach devised by Hancock (8). We do observe a small degree of histone redistribution during our normal isolation procedures, but if divalent cations are excluded from the isolation media, as suggested by Hancock (8), then essentially no intermolecular reorganization of histone occurs (10). Reorganization with respect to strand of a given histone may conceivably occur but this will not affect the analysis, as outlined in Fig. 1 and Table 1. All experiments to be described utilize the Hancock isolation procedure.

For this experimental technique to be successful it is important that the pulse and chase parts of the experiments do indeed encompass, rather precisely, one cell generation, and that DNA synthesis continues unaffected by the various experimental manipulations. In all the experiments to be described we have checked these parameters by analyzing the efficiency and time dependence of incorporation of density label into DNA. Samples of unfixed chromatin were mixed with CsCl solutions so that DNA of different densities could be separated. A typical example is shown in Fig. 3 for Exp. 1 of Table 1. We see that after one generation of incorporation of IdUrd, essentially all the DNA is in the denser peak; after one generation of subsequent growth in fresh medium we see that 50% of the DNA is of normal density and 50% is of the higher density. A second generation produces 75% of the DNA of normal density and 25% of high density. This is precisely the distribution expected for DNA that was labeled with IdUrd for one generation and chased for either one or two generations. Evidently these cells grow well in the presence of 10^{-4} M IdUrd, and there is no significant effect on the cell generation time.

Having established the basic technology, we were in a po-

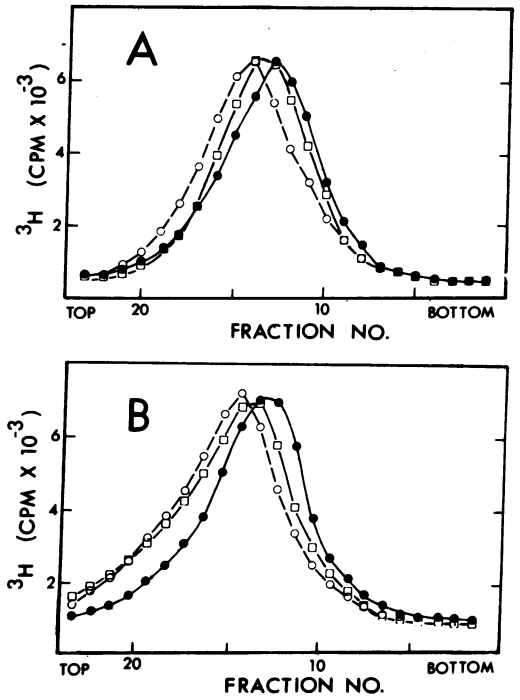

FIG. 4. Distribution of [^{3}H]lysine and [^{3}H]tryptophan relative to density-labeled DNA during Exp. 1. (A) The distribution of [^{3}H]lysine in both histones and nonhistone protein after a pulse of IdUrd + [^{3}H]lysine (●); after one generation chase (□); after two generations chase (○). (B) The distribution of [^{3}H]tryptophan in nonhistone protein during the pulse of IdUrd + [^{3}H]tryptophan (●); after one generation chase (□); after two generations chase (○). All samples were aligned with respect to a fixed chromatin sample containing [^{14}C]thymidine (not shown).

sition to perform the three types of experiments discussed and analyzed in detail in Table 1. The raw data, for the first experiment, uncorrected for nonhistone protein distribution, are shown in Fig. 4; application of the correction leads to the data of Fig. 5. In the first experiment both [^{3}H]lysine and IdUrd were present for one generation. As shown in Figs. 4 and 5A, after this pulse period both the [^{3}H]lysine and nonhistone proteins are distributed fully on dense DNA as expected, and as such this experiment serves as a control for the position of dense nucleoprotein within the CsCl density gradient. After one full cell cycle in the absence of either label, the [^{3}H]lysine is distributed uniformly, midway between normal density and the dense nucleoprotein. This is the expected distribution for a mixture of equal numbers of molecules of dense and normal density material, both containing equal amounts of [^{3}H]lysine. After a second generation the [^{3}H]lysine distribution is shifted towards lower density but, nonetheless, it is still situated slightly to the dense side of a normal density nucleoprotein. This is expected for a population of three parts normal density nucleoprotein and one part dense. If the mode of distribution proposed by Tsanev and Russev (2) were correct, the histone should have remained associated with dense DNA throughout all phases of the experiment (see Table 1, Model B-3). In the second experiment (Fig. 5B) the density label was added for one generation and then replaced by [^{3}H]lysine for one cell generation. Immediately after the [^{3}H]lysine pulse, 50% of all the DNA molecules are density-labeled. [^{3}H]Histones are distributed halfway between the positions of normal density and dense chromatin. After a chase period the histone distribution shifts to lighter regions; however, even after a second

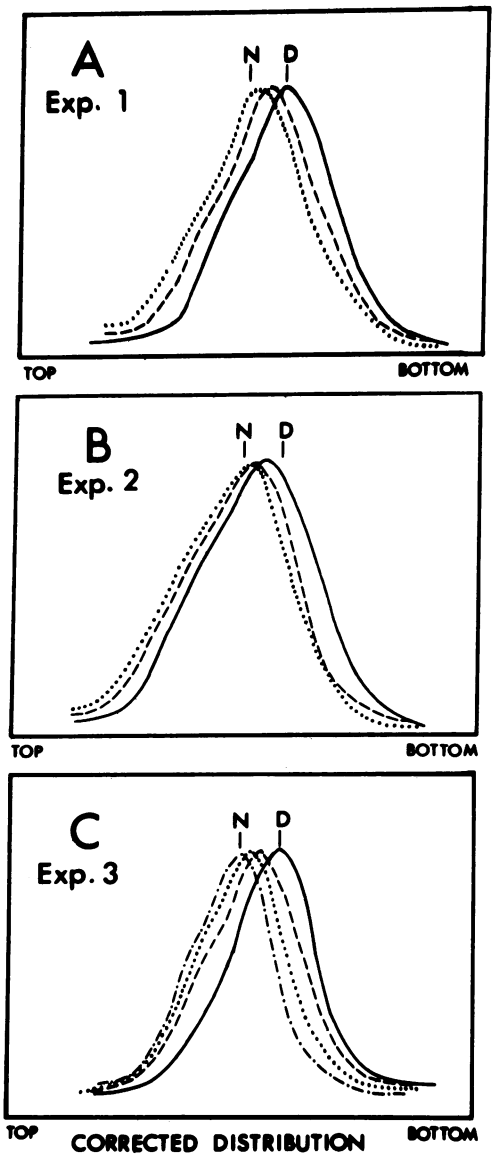

FIG. 5. Distribution of [^{3}H]histone with respect to density-labeled DNA. (A) Corrected histone distribution during Experiment 1 (see *text*). After a 16-hr pulse of IdUrd + [^{3}H]lysine (—); after 16-hr chase (- - -); after 32-hr chase (• • •). (B) Corrected histone distribution during Experiment 2 (see *text*). After 16-hr pulse in IdUrd, followed by 16-hr chase in [^{3}H]lysine (—); after 16-hr chase (- - -); after second 16-hr chase (• • •). (C) Corrected histone distribution during Experiment 3 (see *text*). After 16-hr [^{3}H]lysine pulse, (• - • -); after 16-hr chase in IdUrd(—); after 16-hr chase, no label (- - -); after final 16-hr chase (• • •). All samples were aligned with [^{14}C]thymidine in control fixed chromatin. N refers to the peak of [^{3}H]histone distribution on chromatin containing no density label. D refers to the peak of [^{3}H]histone distribution on chromatin, the DNA molecules of which all contain one strand that is fully density-labeled with IdUrd.

chase period, normal density distribution of [^{3}H]histone is not fully attained and we conclude that we are seeing a continual dilution of radiolabeled histones associated with the density-labeled DNA molecules.

The third experiment (Fig. 5C) consisted of an initial pulse of [^{3}H]lysine, after which the histone is obviously on normal density nucleoprotein, and as such, this forms a control for the distribution of normal density nucleoprotein. We next initiated a chase in which the radiolabel was replaced

by IdUrd (at this point the histone is completely shifted onto dense DNA). Additional chase periods ensued, both of one cell generation in time span. After the first such chase in the total absence of label, the radioactive histone is found distributed halfway between normal and dense DNA molecules, and after the second chase period 75% of the [^{3}H]histone is on DNA molecules of normal density.

The entire approach was repeated with [^{3}H]arginine, and strictly analogous data were obtained. We conclude that the arginine-rich histones deposit in the same manner as the lysine-rich histones. These results are consistent with a random mode of deposition as outlined in Table 1.

DISCUSSION

Utilizing the experimental protocol we have devised, we conclude that histones become associated with newly synthesized DNA in a random manner. Further, as histones do not turn over significantly (7), we conclude that pre-existing histones are likewise randomly disposed onto the daughter DNA molecules during replication. The results also preclude a model whereby an incoming histone could interact with either DNA strand at random, but once this interaction is established the DNA and histone could not separate. This conclusion is in contradiction to that obtained by Tsanev and Russev (2). However, these authors used a heterogeneous population of dividing and nondividing cells (regenerating liver) of uncertain frequency and time length of generation. Their isolation procedures may well have been conducive to reorganization, and rigorous attempts were not made to account for the distribution of nonhistone proteins. Furthermore, their analysis involved the production of small nucleoprotein molecules, which tend to sediment with disassociated histones and nonhistone protein.

These conclusions indicate that histones recognize the DNA molecule as a whole, rather than an aspect specifically of one or the other strand, and that at replication a pre-existing histone or group of histones has an equal chance of becoming connected with either of the two daughter DNA molecules. This does not appear to be an unreasonable conclusion in view of what is known of histones in their role as chromosomal structural proteins. They can generate similar structures with DNA from widely diverse organisms, though they themselves change but little. Furthermore, specific information encoded in histone molecules for the specific deposition of new histone appears unlikely since a single mammalian protamine species can be correctly replaced by histones after fertilization.

The results do not exclude the possibility that histones are deposited in a highly organized manner at the replication fork, and that they subsequently randomize.

We wish to thank Dr. Charles S. Swenson for the use of his Dupont Curve Analyzer, without which this work could not have been completed. We thank our colleagues within this laboratory for their advice and the pleasant environment in which to work. This study was supported by the USPHS Grants nos. Ca-10871 and GM-10871. A preliminary report on this work was presented at the 1st Annual Florida Colloquium on Molecular Biology.

1. Prescott, D. M. & Bender, M. A. (1963) *Exp. Cell Res.* **29**, 430 (VIII).
2. Tsanev, R. & Russev, G. (1974) *Eur. J. Biochem.* **43**, 257–263.
3. Hancock, R, (1970) *J. Mol. Biol.* **48**, 357–360.
4. Jackson, V. & Chalkley, R. (1974) *Biochemistry* **13**, 3952–3956.
5. Brutlag, D., Schlehuber, C. & Bonner, J. (1969) *Biochemistry* **8**, 3214–3218.
6. Varchavsk, A. J. & Georgiev, G. P. (1972) *Biochim. Biophys. Acta* **281**, 669–674.
7. Balhorn, R., Oliver, D. & Chalkley, R. (1972) *Biochemistry* **11**, 1094–1099.
8. Hancock, R. (1974) *J. Mol. Biol.* **86**, 649–663.
9. Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285.
10. Jackson, V. & Chalkley, R. (1975), *First Annual Florida Colloquium on Molecular Biology.*