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Histone Segregation on Replicating Chromatin†

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ABSTRACT: We have reinvestigated the mode of segregation of preexisting histones onto replicating chromosomes. Since our previous data have indicated that only histones H3 and H4 do not appear to move from their association with the DNA strand with which they are bound until the next round of replication, we have concentrated our attention on these two histones. The strategy we have employed involved density labeling of DNA and radiolabeling of the histones of interest. Subsequently, we followed the association of histones and DNA during further rounds of DNA replication. One can make predictions concerning the nature of the association between specific histones and particular DNA strands depending on the mode of deposition. The results have confirmed our previous findings that histones segregate randomly. The possibility that such a result is a consequence of turnover of radiolabel in non-histone proteins and subsequent reutilization for histone synthesis has been tested directly. This process appears to be occurring to only a very limited extent. The implications of these conclusions for chromatin structure and gene control are discussed.

The mechanism whereby chromosomal proteins are deposited on replicating DNA molecules is of great interest. This is not only because of the intrinsic value of the description of a complex biological process but also because it appears that a specific arrangement of proteins is found in the region of transcriptionally active genes and the possibility exists that a part or all of this organization may be established during the replicational process.

The way in which old (preexisting) histones are distributed to daughter DNA strands can take one of three general pathways. One pathway involves unilateral distribution, in which all the preexisting histones are associated with only one of the two daughter strands. Obviously, several variations within this theme are possible; for instance, old histones could be associated exclusively with either the leading or the lagging side of the replication fork. These possibilities are documented in Table I. This general method of segregation is sometimes described as conservative segregation. Bilateral distribution, in which the preexisting histones ahead of the replication fork become associated with both daughter DNA molecules, constitutes the remaining two pathways since this may occur in two general ways, either in a random manner or in an ordered manner. In the latter case, the histones would show an affinity for a particular DNA strand.

We have attempted to address this question using an experimental protocol in which histones were labeled with [^{3}H]lysine and DNA was density labeled with iododeoxyuridine during generation-long pulse periods (Jackson et al.,

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1975a,b). Under these conditions, we observed a random association of histones and new DNA, leading us to conclude either that histones are deposited in an ordered manner or that they subsequently move to generate an overall random mode of association. Indeed, during the last few years, it has become apparent that three of the five histones, namely, H1, H2A, and H2B, are much more mobile than was previously thought (Jackson & Chalkley, 1981a,b; Jackson et al., 1981; Louters & Chalkley, 1984). In the presence of incoming, newly synthesized histone, H1 is so mobile that it probably cannot be thought of as binding any particular site on the chromatin but rather provides a general polycation cloud. Histones H2A and H2B seem to be able to exchange with intranucleosomal H2A and H2B with considerable facility both in vitro and in vivo, though whether this is a generalized or a spacially localized effect is not known.

In contrast, histones H3 and H4 do not appear to move from strand to strand in this manner. Indeed, we have recently reported that these histones remain in association with the DNA strand upon which they were deposited until the next round of DNA replication (Jackson & Chalkley, 1981b). Since the position of these histones almost certainly dictates that of the other three histones, it is of considerable interest to determine the nature of the deposition of histones H3 and H4. Our previous studies of deposition assayed for histones considered as a group (total ^3H label), rather than following the behavior of individual histones themselves. We have therefore repeated our earlier studies taking advantage of advances in technology which enable us to follow the behavior of individual histones. In addition, we have also tested whether turnover of non-histone proteins during chase periods might provide a significant source of radiolabel for histones, thus giving an appearance of random association where none existed. We have found that the level of such turnover and reutilization is very low and would not be a source of ambiguity in the analysis of the data.

Experimental Procedures

Labeling of Cells. Logarithmically growing hepatoma tissue culture cells (HTC cells) were maintained between 2×10^5 and 4×10^5 cells/mL in Swims 77 medium supplemented with 5% newborn calf/5% calf serum in suspension culture. In a typical experiment, cells were collected after each generation (18 h), a sample was retained, and the remaining cells were resuspended in fresh medium containing any required additions. Density labeling of DNA was achieved by growth of the cells in the presence of 0.0001 M iododeoxyuridine (IdUrd) for 18 h. HTC cells are one of the few cell lines which incorporate and maintain this base analogue within the DNA for several generations without inhibiting cell growth or DNA replication. Histones were labeled with [^3H]lysine during an 18-h exposure to 1 mCi of [^3H]lysine (40 Ci/mmol, Schwarz/Mann). At each time point in the experiments to be described below, 100 mL of cells would be reserved for an assay of the level of IdUrd incorporation, 400 mL of cells would be collected for fixation and density gradient analysis, and the remaining 500 mL of cells would be centrifuged and resuspended in the required medium as described in the text.

Density Gradient Analysis of IdUrd Incorporation into DNA. Chromatin was prepared by standard procedures (Jackson et al., 1975c) including a final wash with deionized water in order to cause swelling of the nucleoprotein and facilitate efficient shearing. The material was sheared in a Virtis homogenizer at full speed for 30 s and then centrifuged at 27000*g* for 10 min to remove membraneous debris. The supernatant from this last step, the soluble chromatin, was adjusted to a final volume of 7 mL in a solution containing 6.8 g of CsCl and 0.1 mL of 1.0 M tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl). The high ionic strength dissociates protein from DNA so that the density of DNA alone can be measured. Centrifugation was at 43 000 rpm for 48 h at 4 °C in a Beckman 50 rotor. The distribution of DNA on these gradients was assayed by the absorbance at 260 nm after 15-drop fractions were collected. If the distribution of IdUrd in DNA on these gradients indicated that for some reason the cells were not maintaining an 18-h cell cycle, all experiments following the protocols of Figure 1 were terminated, and the cells were not used. Only when precise generation times were maintained throughout the entire experiment did we analyze the histone distribution.

Preparation and Fractionation of Chromatin from Fixed Cells. Cells were harvested by centrifugation (2000 rpm/5 min) and resuspended in Dulbecco's modified Eagle's medium containing 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, and 1% formaldehyde. The cells were stirred for 2 h at 4 °C, then collected by centrifugation at 2000*g* for 10 min, and washed twice in the incubation medium lacking formaldehyde. The cells were then resuspended in the same medium and stirred for 18 h at 4 °C. The cells were then washed 3 times in 1% Triton X-100, 0.25 M sucrose, and 10 mM $MgCl_2$. Finally, the chromatin was washed twice with 4 M guanidine hydrochloride in 10 mM ethylenediaminetetraacetic acid (EDTA) to remove tightly bound protein which is not cross-linked to DNA by the formaldehyde treatment. This wash treatment does not remove histones as they are quantitatively cross-linked to the DNA (Jackson, 1978; Jackson & Chalkley, 1981a). The chromatin was then mixed with unlabeled, normal density chromatin (also prepared from fixed cells) in a 1/5 ratio (an excess of unlabeled chromatin). This mixture was then sonicated in 2-mL samples for 60 s at setting 3 of a Branson sonifier. The sample was then centrifuged at 27000*g* to remove insoluble material. The supernatant consists of chromatin which contains DNA of an average size of approximately 1 kilobase pair. The chromatin is added to 1.2 g of CsCl and the volume adjusted to 4.6 mL with 4.0 M guanidine hydrochloride in 10 mM EDTA. The samples were then centrifuged to equilibrium at 35 000 rpm for 72 h at 4 °C in a Beckman SW56 rotor.

Reversal of Cross-Links and Gel Electrophoresis. After centrifugation, it is necessary to reverse the histone cross-links before analyzing their distribution relative to DNA. This is achieved by treating each sample with 20 μL of 2-mercaptoethanol and heating at 100 °C for 30 min. The samples were then dialyzed against two changes of 2 L of 50 mM 2-mercaptoethanol at 4 °C for 24 h. The samples were adjusted to a final 5% glycerol–0.2% sodium dodecyl sulfate (SDS)–50 mM Tris, pH 7.4–0.1% bromphenol blue from a 5× stock. The electrophoresis conditions are a modification of the Laemmli (1970) procedure. The electrophoresis buffer is 0.1% SDS, 0.025 M Tris, and 0.2 M glycine, pH 8.3, and the separating gel is 18% acrylamide, 0.09% methylenebis(acrylamide), and 0.75 M Tris (pH 8.8). Electrophoresis was at 150 V for 18 h at 4 °C, after which the gels were stained in 0.1% Coomassie brilliant blue–50% methanol–10% acetic acid for 12 h, destained in 40% methanol–10% acetic acid, scanned in an RFT-II scanning densitometer, and fluorographed (Laskey & Mills, 1979).

Analysis of Radiolabel Incorporated into Histones from Protein Turnover. HTC cells were synchronized into the G1 phase of the cell cycle by mitotic block with 0.25 mM colcemid for 8 h followed by selective detachment (Moore et al., 1979).

Experimental Protocols

	1st generation	- +^{3}H-lysine
	2nd generation	- chase ^{3}H-lysine
Expt. 1		+Iudr
	3rd generation	- chase ^{3}H-lysine and Iudr
	4th generation	- chase ^{3}H-lysine and Iudr
	2nd generation	- +^{3}H-lysine + Iudr
Expt. 2	3rd generation	- chase ^{3}H-lysine and Iudr
	4th generation	- chase ^{3}H-lysine and Iudr
	2nd generation	- + Iudr
	3rd generation	- chase Iudr
Expt. 3		+ ^{3}H-lysine
	4th generation	- chase Iudr and ^{3}H-lysine
	5th generation	- chase Iudr and ^{3}H-lysine

FIGURE 1: Experimental protocols for analysis of histone distribution on daughter strands after DNA replication. The first two cell generations are used for labeling protocols using either [^{3}H]lysine, iododeoxyuridine (Iudr in figure), or a combination of both labels as indicated. Later generations involve chases of both radiolabel and density label.

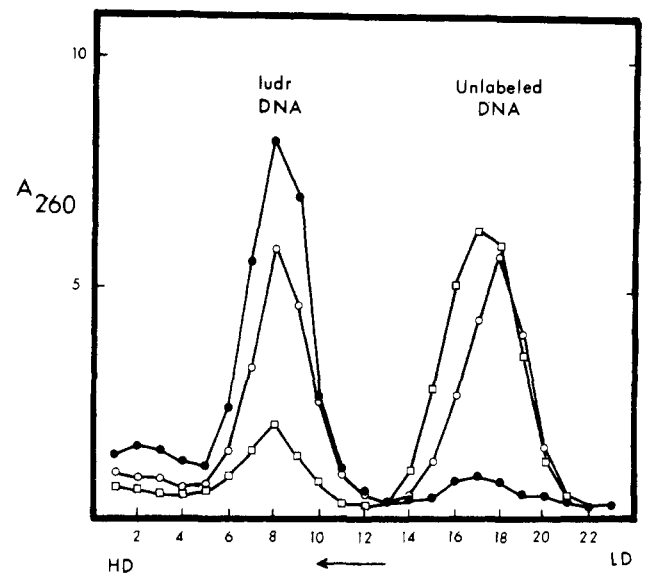

FIGURE 2: Analysis of IdUrd incorporation into DNA. Cells were labeled according to the protocol of experiment 2 (Figure 1) and aliquots collected at the indicated time points. Chromatin was isolated and centrifuged to equilibrium on a CsCl density gradient. At these high salt concentrations, histones are released from the DNA, allowing the DNA density to be determined. HD and LD refer to the regions of high and low density, respectively.

The detached cells were washed in Swims S-77 medium containing 5% newborn calf/5% calf serum and resuspended in this medium for incubation in either the presence or the absence of 10 μCi mL [^{3}H]lysine. Cells were harvested by centrifugation at low speed, and the pellets were rapidly frozen. The cells were disrupted by sonication in H_2O (2 mL), and H_2SO_4 was added to a final concentration of 0.2 M. The solutions were centrifuged at 27000*g* for 10 min, and the acid-soluble proteins in the supernatant were precipitated with 7 volumes of acetone at −20 °C for 18 h. The precipitated protein was collected and dissolved in 8 M urea, 0.9 N acetic acid, 0.5 M 2-mercaptoethanol, and 0.25% methyl green. These samples were then analyzed by electrophoresis in gels containing 12% acrylamide, 0.08% bis(acrylamide), 0.9 N acetic acid, 8 M urea, and 0.45% Triton X-100 for 20 h at 250 V at room temperature (Zweidler, 1978). The gels were stained with Coomassie blue as described above, and gel strips were cut out and applied to the top of a second gel containing SDS (see above) for electrophoresis in a second dimension (Jackson, 1978). A microdensitometric scan was obtained for histones H2A and H2B both from the stained gel and from the fluorogram to determine the amount of [^{3}H]lysine incorporated into histone from protein turnover.

RESULTS

Cell Generation Times Can Be Effectively Defined. In order to study the mode of histones deposition with respect to DNA strand, we have utilized the labeling protocols outlined in Figure 1. Unambiguous conclusions from this approach demand that the labeling periods are as close to unit-cell generation times as possible. We have checked this in all experiments reported in this paper. Typical results of such a check are shown in Figure 2. In this experiment, HTC cells were labeled with [^{3}H]lysine and IdUrd for one complete generation. Subsequently, both labels were removed and the cells resuspended in fresh unlabeled medium for a full generation, at which time some of the cells were collected. Finally, the remaining cells were harvested after one more cell cycle was complete. The density of the DNA at the various times of collection was assayed by CsCl density gradient centrifugation. As shown in Figure 2, almost all of the DNA has an increased density after growing for one complete generation in IdUrd, reflecting the incorporation of density label into one strand. An underestimation of the cell generation time would have been reflected in the presence of DNA of normal density; likewise, DNA of greater density would reflect an overestimate of the period of the cell cycle. The correct timing of the next cell cycle is reflected in the presence of two peaks of DNA on the density gradient each of which contains the same amount of material. Finally, the last cell cycle leaves us with one-fourth of the DNA in the hybrid density position and three-fourths of the DNA of normal density.

Histone Distribution with Respect to DNA Strand. It is possible to assign the mode of histone distribution (for those histones which do not migrate by performing three experiments in which the relative times of generation-long pulses of [^{3}H]lysine and IdUrd are varied as outlined in Figure 1. Depending on the mode of segregation, specific predictions can be made concerning the association of radiolabeled histone and density-labeled DNA. These predictions are summarized in Table I. See also Figure 8 for an illustration of three specific models and the predicted results by these experimental procedures.

In the first of the three experiments, HTC cells were first labeled for one generation with [^{3}H]lysine. The radiolabel was then replaced with IdUrd for one cell generation to density label one strand of all the daughter DNA molecules in all replicating cells. The density label was then removed, and the cells were allowed to proceed through one and then a second cell generation. Samples of cells were collected at the termination of each of the generation periods. These cells were

Table I: Mechanisms of Histone Deposition[a]

	expt 1				expt 2				expt 3				
generation:	1	2	3	4	1	2	3	4	1	2	3	4	5
Unilateral													
(1) new histone on lagging DNA strand	N	D	N	N	–	D	D	D	–	–	$^1/_2$D	N	N
(2) new histone on leading DNA strand	N	D	N	N	–	D	D	D	–	–	$^1/_2$D	N	N
(3) new histone on newest DNA strand	N	D	N	N	–	D	N	N	–	–	D	$^1/_2$D	$^1/_2$D
(4) new histone on oldest DNA strand	N	D	D	N	–	D	D	N	–	–	N	N	N
Bilateral													
(5) new histone on new DNA strand	N	D	N	N	–	D	D	D	–	–	$^1/_2$D	N	N
(6) new histone on old DNA strand	N	D	D	N	–	D	N	N	–	–	$^1/_2$D	$^1/_2$D	N
(7) new histone on alternating new–old DNA strand	N	D	D	N	–	D	D	N	–	–	$^1/_2$D	$^1/_2$D	$^1/_2$D
(8) new histone on new DNA strand (switch daughter strands)	N	D	D	N	–	D	N	N	–	–	$^1/_2$D	$^1/_2$D	N
(9) new histone on old DNA strand (switch daughter strands)	N	D	D	N	–	D	D	D	–	–	$^1/_2$D	N	N
(10) new histones on both new and old DNA strands (*randomly*)	N	D	$^1/_2$D	$^1/_4$D	–	D	$^1/_2$D	$^1/_4$D	–	–	$^1/_2$D	$^1/_4$D	$^1/_8$D
(11) new histones on both new and old DNA standards randomly; however, histones are ordered with respect to each other, i.e., new–old–new–old etc.	N	D	$^1/_2$D	$^1/_4$D	–1	–D	$^1/_2$D	$^1/_4$D	–	–	$^1/_2$D	$^1/_4$D	$^1/_8$D
results	N	D	$^1/_2$D	N	–	D	$^1/_2$D	N	–	–	$^1/_2$D	N	N
RSA	1.0	9.5	2.8	1.5		11.1	2.6	1.3			3.0	1.0	1.1

[a] In this table, we have illustrated the theoretical results of 11 possible models of histone segregation if the protocols of Figure 1 are followed. Also tabulated are the results actually obtained in the experiments themselves. D denotes that the radiolabeled histones are associated with DNA molecules which contain one density-labeled strand. L denotes that the radiolabeled histones are associated with DNA which is totally lacking IdUrd. $^1/_2$D indicates that the radiolabeled histones are associated with equal numbers of DNA molecules of both normal and increased density. $^1/_4$D indicates that the radiolabeled histones are equally distributed between one density-labeled and three normal density DNA molecules. The relative specific activity (RSA) is defined as the ratio of fluorographic intensity to staining intensity normalized to a value of 1 for the point at the center of the normal density of chromatin in the gradient. If the RSA of histones which are fully associated with IdUrd-labeled DNA is known, it is possible to calculate what new value for the RSA would be found if the histones were equally distributed between dense and normal DNA molecules after an additional generation of growth in the absence of IdUrd. For instance, if the fluorographic intensity in the dense and light regions were 5 and 3 units, respectively, and if the staining intensities were 1 and 6, respectively, we find that the specific activity in the dense region of the gradient is 5/1 = 5 and in the position of normal density it is 3/6 = 0.5, and thus the RSA = 5/0.5 which is 10. Now assume that during the next full generation the histones distribute themselves equally between dense and light DNA molecules. Half of the radiolabeled which was previously dense (2.5) shifts from dense to light. The distribution of staining material is unchanged because of the 5-fold excess of unlabeled chromatin maintained as marker. Thus, the specific activity in the dense region is (5 − 2.5)/1 = 2.5, and in the normal density region, it is (3 + 2.5)/6 = 0.9 so that an RSA = 2.5/0.9 = 2.7 is obtained. We emphasize that a shift of histones from an association exclusively with dense DNA to a 50% association will lead to a shift in RSA from 10 to 2.7 and not to a value equal to 5.

then fixed with formaldehyde as described previously (Jackson & Chalkley, 1981b). This prevents any nucleoprotein migration during the subsequent cell disruption (Jackson & Chalkley, 1981a). Nuclear material was isolated and fractionated on a CsCl density gradient, together with a constant 5-fold excess of non-density-labeled chromatin. The excess unlabeled chromatin is needed to establish the location of normal density material within the gradient. By maintaining a constant ratio of 5/1 for unlabeled DNA/density-labeled DNA within the chromatin, we can quantitate any change in the distribution of labeled histones associated with the DNA during the various pulses and chases. Samples from each point in the gradient were collected, the cross-links were reversed, and histones were isolated for analysis by SDS gel electrophoresis. In this way, we could assay for the distribution on the gradient not only of the non-density-labeled chromatin (shown by the position of the Coomassie staining of the histones from the added excess of control chromatin) but also of the radiolabeled histones (indicated by the results of the fluorographic analysis of the SDS gels of the gradient fractions). The results are presented in the form of a ratio of the intensity of histones H3 and H4 in a fluorograph to the intensity of stain in a Coomassie-stained gel for these same histones in each fraction from the density gradient.

The results are shown in Figure 3. After the first generation of growth in the presence of [^{3}H]lysine but in the absence of density label, the labeled histone is distributed uniformly over the normal density chromatin peak. As expected, the ratio of fluorographic intensity to stain intensity [the relative specific activity (RSA)] is constant throughout the gradient. The next generation of growth was in the presence of IdUrd, and one strand of each daughter DNA molecule is density labeled. As a consequence, the labeled histone is distributed toward the dense side of the main (control density) chromatin peak, showing a relative specific activity of 10. These two experiments provide assurance that the CsCl density gradient is indeed resolving chromatin species on the basis of density. The next phase of the experiment involves growth in the absence of any label. As shown in panels 3 and 4 of Figure 3, after only one generation in the absence of density label the histones synthesized in the first phase of the experiment are associated to a reduced degree with the density-labeled DNA strand, and even less so after the final generation of growth. Upon quantitation of these results, we find that the RSA decreases from a value of 10 when histones H3 and H4 are fully associated with the IdUrd-containing strand to a value of 3 after one generation. This decreases to 1.5 after the final round of growth. Two points emerge from these observations, namely, that the radiolabeled histones do not remain exclusively with the density-labeled DNA strand, thus excluding modes 4, 6, and 8 of Table I, and, further, that the labeled histones do not exclusively become associated with only low-density DNA, thereby excluding modes 1, 2, 3, 5, and 7 of Table I. These conclusions will be further substantiated by the results of the additional experiments described below.

In a second experiment, (Figure 4), both radiolabel and density label were added together for one generation. As expected, the radiolabeled histones are now distributed toward the dense side of the gradient, showing an RSA of 10. Both labels were then removed. In the subsequent chase, the radiolabeled H3 and H4 now show a substantially (but not fully) decreased association with the density-labeled DNA. After first one and then another generation, only a small amount of labeled histone remains associated with the IdUrd-containing DNA. Again, reference to Table I indicates that only the random mode of histone association can fit with these

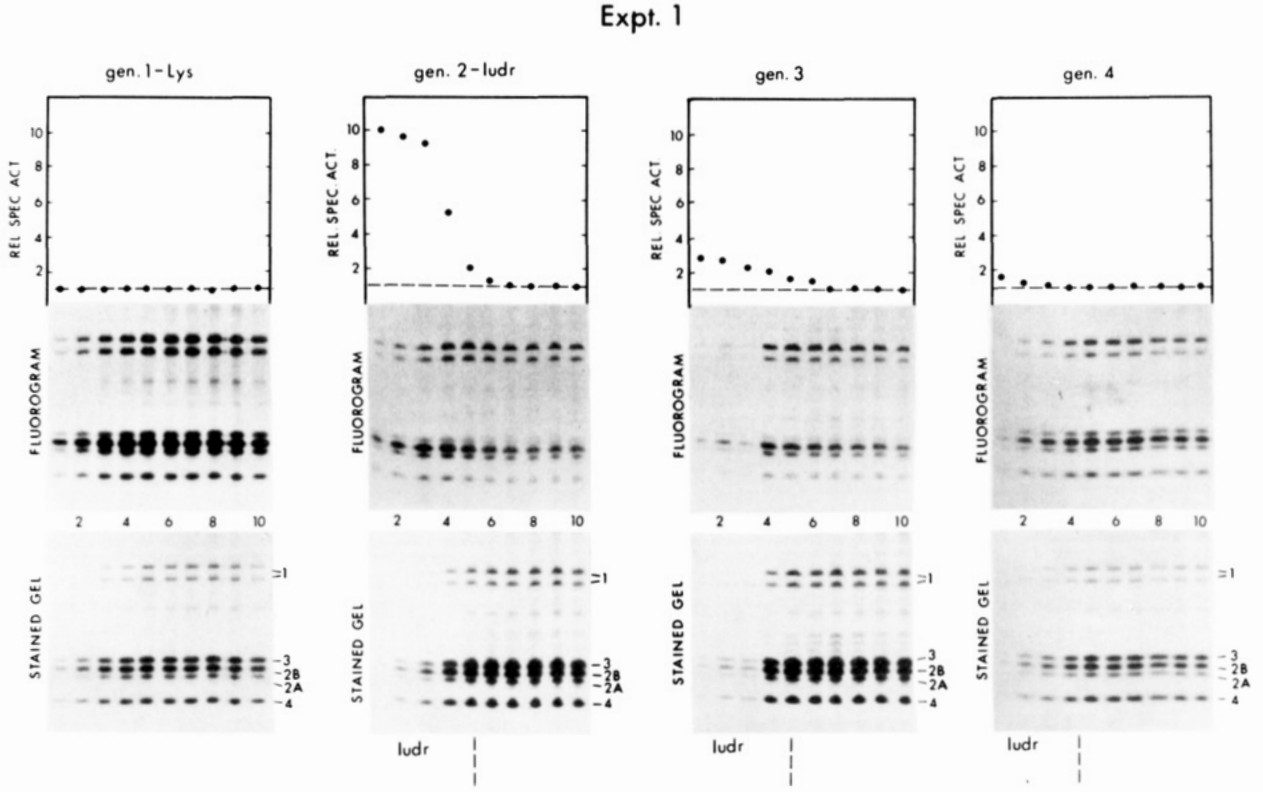

FIGURE 3: Histone distribution results from experimental protocol 1. Cells were labeled with [^{3}H]lysine for one generation. The radiolabel was removed and replaced with IdUrd for the second generation. The density label was removed, and the cells were permitted to grow for two more generation periods. Cells were collected at the end of each generation period. Chromatin was isolated after the cells had been fixed with formaldehyde. An excess of unlabeled chromatin (no radiolabel or density label) was added to the labeled chromatin to maintain a 5/1 ratio (DNA not containing IdUrd/DNA containing IdUrd). Therefore, a 5-fold excess of unlabeled chromatin was added to the cells from the labeling in IdUrd for one generation (all DNA density labeled). However, for the cells from the subsequent chase of one generation (50% DNA not density labeled), we added a 2.5-fold excess of unlabeled chromatin and for the second generation chase a 1.25-fold excess. The chromatin was centrifuged to equilibrium on a CsCl density gradient, fractions were collected, the cross-links were reversed, and the histone distribution was determined by analysis on SDS–polyacrylamide gels. The notation Iudr occurring to the left of the dashed line in each panel indicates the zone in which the large bulk of IdUrd-containing chromatin is found. This is determined, for instance, by the distribution of labeled histone in the panel entitled gen.2-Iudr where all of the histone is necessarily associated with density-labeled strands of DNA. The distribution of bulk histones (indicated by the stained material) reflects the 5-fold excess of non-density-labeled chromatin which is maintained in each sample before centrifugation and which is used as an internal density standard. The relative specific activity (RSA) is the ratio of fluorographic intensity to Coomassie stain intensity normalized to the lowest such ratio across the gradient being set to a unit value.

observations. Since we have previously argued (Jackson & Chalkley, 1981b) that H2A and H2B are capable of a measure of exchange, we have calculated the RSA for H2A and H2B and have obtained exactly the same result as for H3 and H4, indicating that at the moment of replication there is not a process of ordered distribution of H3 and H4 which is overshadowed by a random distribution of H2A and H2B.

In the final experiment (Figure 1, experiment 3), the cells were first labeled with IdUrd. During a second generation, the density label was replaced with [^{3}H]lysine. As shown in Figure 5, analysis of an aliquot of these cells revealed an RSA of ~3, indicating that the radiolabel is associated about equally with both strands of DNA. Subsequent cell cycles reveal very little association of radiolabeled histone with the density-labeled strand synthesized the generation previously. This result is consistent with the random mode of histone deposition though by itself it is not sufficient to exclude other more ordered modes such as 1, 2, 5, and 9 of Table I.

Reutilization of Label during Chase Periods Is Not Substantial. The experiments described above involve extended chase periods. Although histones themselves turn over at an imperceptible rate in growing HTC cells, the possibility remained that a significant proportion of non-histone proteins is turning over during the chase period and released radiolabel is being efficiently reutilized for new histone synthesis. To the extent that it might occur, this could interfere with the interpretation of the mode of histone deposition. Accordingly, we have assayed such turnover and reutilization directly. The strategy involves incorporating [^{3}H]lysine into cell proteins largely in the absence of histone synthesis and measuring its subsequent turnover and incorporation into histones during S phase. This can be done in two ways. Since histone synthesis is reduced to less than 2% of control in Gl-phase cells, it is possible to label cells for 3 h in G1 without any additional intervention before significant histone synthesis occurs. A more extended period of non-histone synthesis can be achieved if hydroxyurea is added for 11 h to synchronized G1 cells. Longer periods of exposure to the drug result in significant loss of cell viability.

Cells were labeled by using both of the protocols outlined above to minimize histone synthesis. Cells were synchronized by selective detachment and allowed to progress into G1. They were then pulsed with [^{3}H]lysine either for 3 h in the absence of additional treatment or for 11 h in the presence of hydroxyurea, after which in both cases they were chased in label-free medium for 22 h during which time both protein turnover and histone synthesis occur. In order to assess the significance of the level of incorporation, we also determined the degree of uptake into histones from the same amount of radioactive precursor when histones are being synthesized vigorously. This was assayed by releasing a separate aliquot of G1-synchronized cells into S phase in medium containing

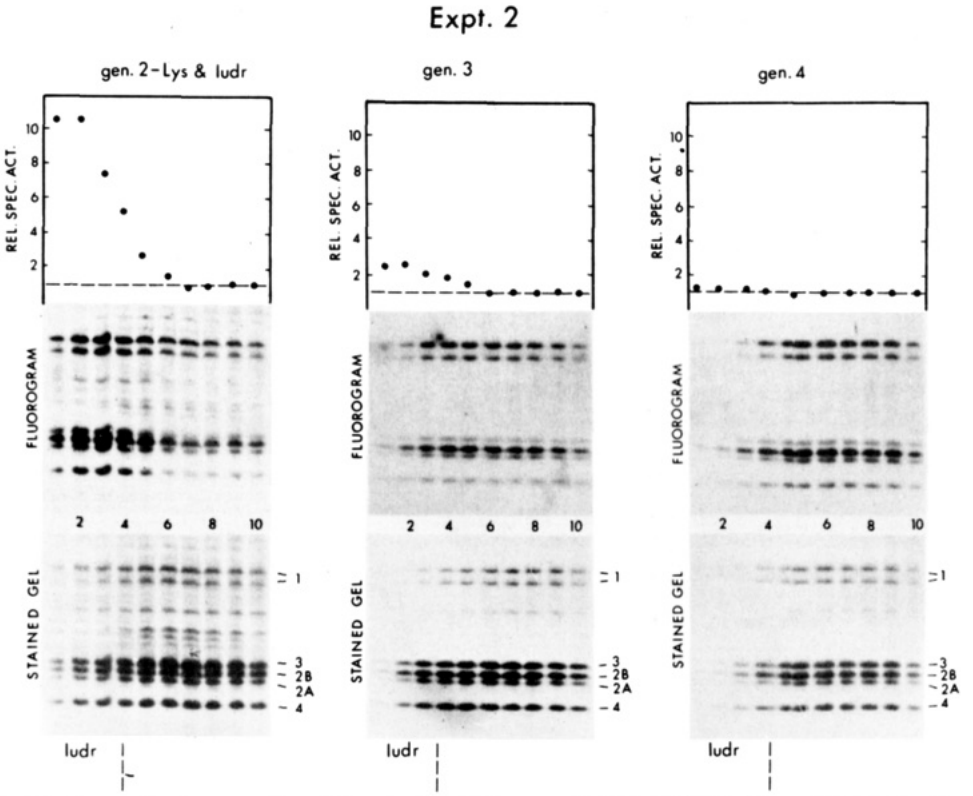

FIGURE 4: Histone distribution results from experimental protocol 2. Cells were labeled with [^{3}H]lysine and IdUrd together for one generation and subsequently chased for up to two generations. The procedures for preparation of chromatin from fixed cells and maintenance of a 5-fold excess of unlabeled chromatin in the subsequent CsCl centrifugation are as described in Figure 3.

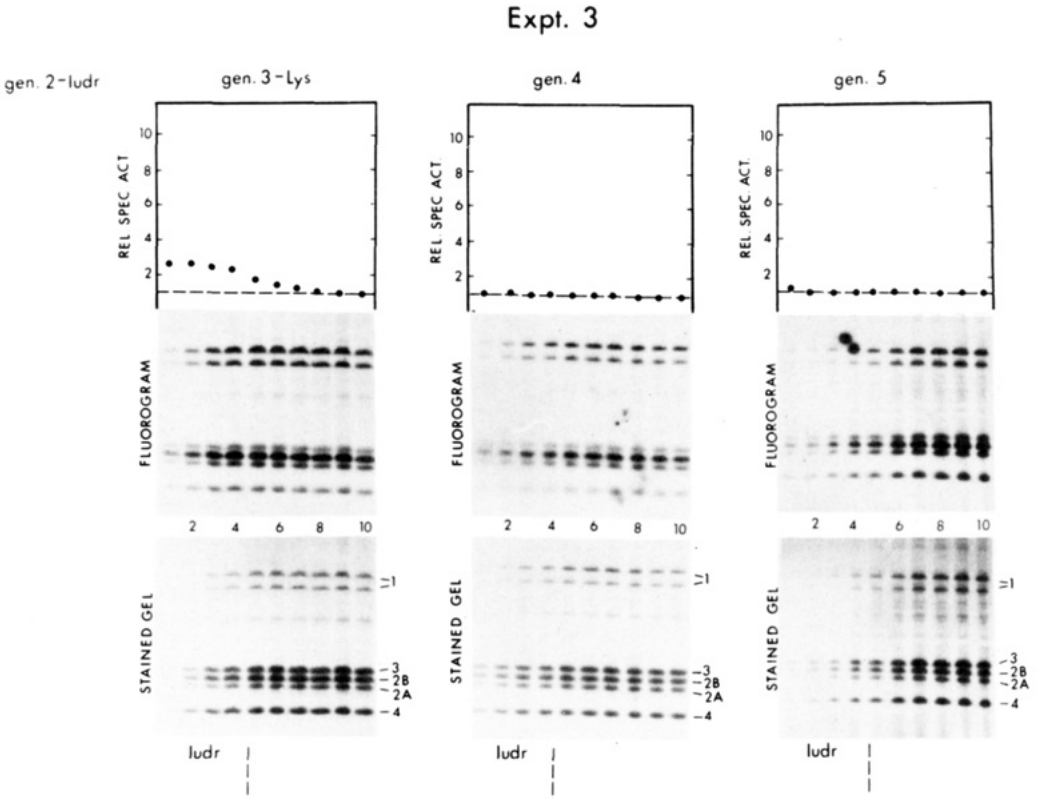

FIGURE 5: Histone distribution results from experimental protocol 3. Cells were density labeled for one generation with IdUrd. This was chased into a second generation in the presence of [^{3}H]lysine, after which the radiolabel was removed and the chase period continued for two further generations. The procedures for analysis of histone distribution were as described in the legend to Figure 3.

radiolabel and measuring the label incorporated into histones. The results of such an analysis are shown in Figure 6 where we see that the reutilization of radiolabel incorporated into non-histone proteins during a 3-h period is only 1% of the radiolabel incorporated directly into histones during a labeling period of comparable time. It is apparent that the extent of turnover and reincorporation into histones is very low indeed. Even when the initial incorporation is 11 h, the incorporation

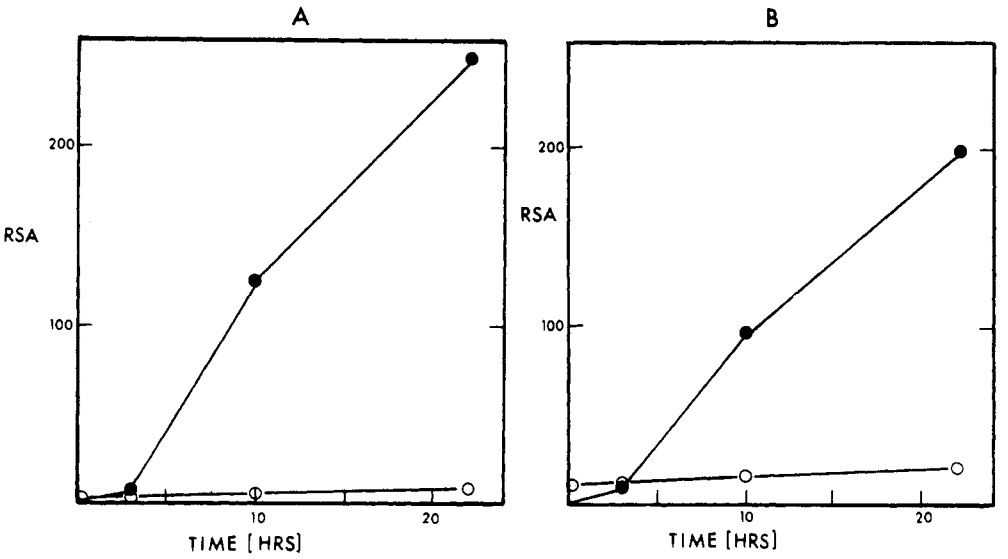

FIGURE 6: Analysis of [^{3}H]lysine uptake into histones from protein turnover. (A) Cells collected in G1. Cells were synchronized into early G1, and one aliquot was pulse-labeled with [^{3}H]lysine for 3 h and chased for 22 h. Samples were collected at the times indicated and histone specific activities determined (○). A second, equal aliquot was grown in the presence of [^{3}H]lysine for the same 22-h period with samples collected at the times indicated (●). (B) Cells collected in G1 and exposed to 5 mM hydroxyurea for 11 h. The G1 cells were divided into two equal samples. One was incubated with [^{3}H]lysine for 11 h and then chased in the absence of both radiolabel and hydroxyurea for 22 h. Samples were collected at the times indicated for assays of histone specific activity (○). The second sample was incubated in hydroxyurea for 11 h. The hydroxyurea was then removed, and these cells were grown in the presence of [^{3}H]lysine for 22 h (●). After completion of a pulse or chase, the cells were disrupted by freezing and subsequently extracted with 0.4 N sulfuric acid. The soluble material was analyzed by two-dimensional electrophoresis (see Experimental Procedures) and the specific activity of histones H2B and H1 determined from the ratio of fluorographic intensity to staining intensity (Jackson & Chalkley, 1985). All specific activities were normalized to a value of 1.0 assigned to the value observed for the 3-h pulse in G1 (the lowest value found in these experiments). The results are presented as relative specific activity values at various times in the chase.

of label into histones from direct uptake and synthesis is 40-fold greater than that from turnover and reutilization. Interpolation of the fraction of radiolabel so reutilized after a pulse of 20 h (one generation) can be obtained by extrapolating the values for reutilization after 3 and 11 h to that expected after 20 h as shown in Figure 7. We find that no more than 4% of the radiolabel previously in non-histone proteins finds its way into histone during a generation-long chase period.

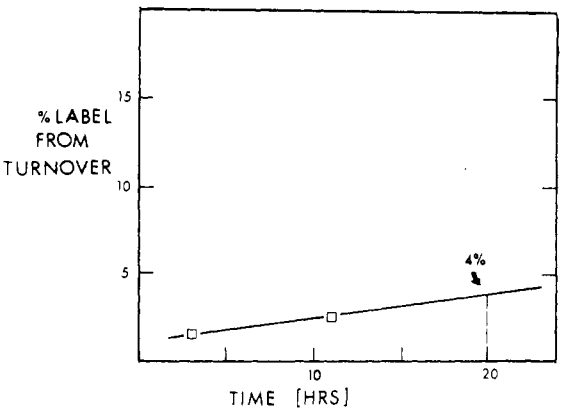

FIGURE 7: Determination of amount of label from non-histone protein turnover incorporated into histone during a one-generation chase period when the non-histone proteins were labeled for a generation time period. The experimentally determined percent conversion of label during a 20-h chase period from non-histone proteins to histones following 3- and 11-h labeling periods was determined from the slope of the lines in the data of Figure 6 and is plotted. Linear extrapolation indicates that after a 20-h pulse about 4% of the label in non-histone protein will be reutilized for histone synthesis during a 20-h chase period in the absence of radiolabel.

Discussion

Studies on the mode of segregation of histones reported in this paper indicate that histones are distributed randomly to daughter DNA strands during replication. Since these studies reflect the positions of histones over many hours, they are only valid if histones do not move from strand to strand during this time period. Obviously, if any histones do migrate, then an apparent random mode of association will be an unavoidable consequence. During the last few years, it has become apparent that some histones do indeed migrate with some facility (Jackson & Chalkley, 1981a,b; Jackson et al., 1981). Thus, we cannot draw any conclusions from these results concerning the mode of segregation of histones H1, H2A, and H2B. However, previous studies have shown that essentially all of new H1 and much of new H2A and H2B are not deposited at the replication fork, thereby demanding that most of the complement of these three histones at the replication fork comes from histones other than those synthesized immediately before, thus implicating substantial histone migration at this time.

In contrast, there is direct evidence not only that histones H3 and H4 are deposited at the replication fork but also that they remain associated with the DNA strand with which they are associated until the next passage of the replication fork (Jackson & Chalkley, 1981b). Accordingly, the basic criteria are met which permit us to pose legitimate questions about the mode of deposition of histones H3 and H4. On the basis of the observations described in this report, we conclude that both preexisting histones H3 and H4 ahead of the replication fork and newly synthesized histones H3 and H4 are deposited randomly with respect to DNA strand. Possibly the clearest way to interpret this result is to conclude that each H3 and H4 in the proximal nucleosome ahead of the replication fork has an equal chance of becoming bound to either daughter DNA strand. The other strand is presumably free to become associated with incoming newly synthesized H3 and H4. We are not in the position to make any assessment as to whether the proximal nucleosome disintegrates or is passed intact during replication. Such a role for the arginine-rich histones is not inconsistent with a host of previous reports (Simon et

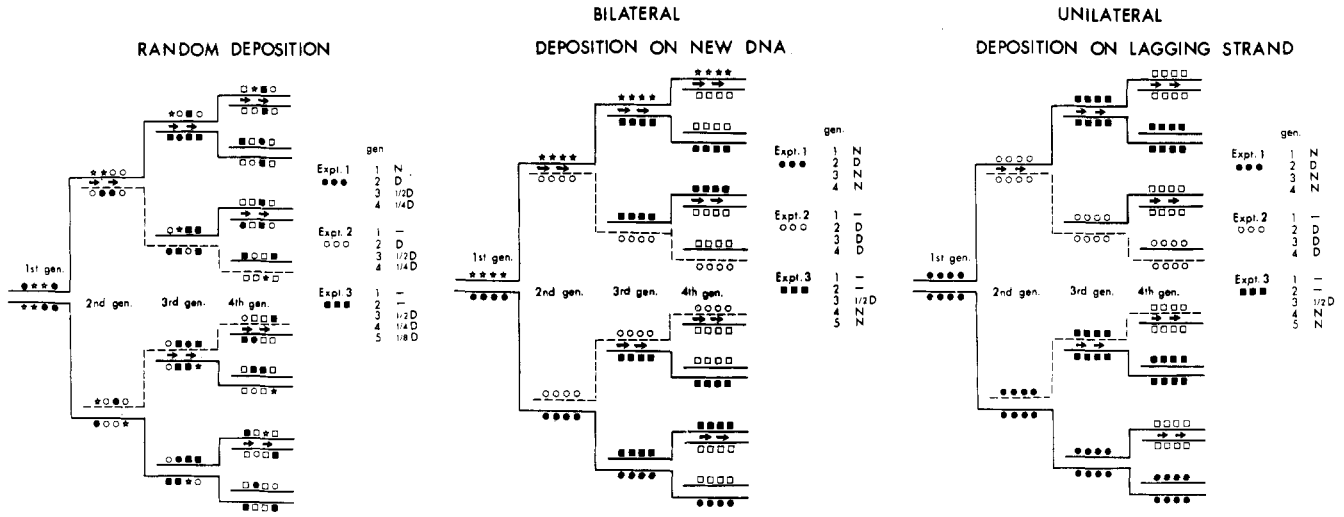

FIGURE 8: Illustration of three models for histone deposition on replicated daughter DNA strands. Experimental support for these models is as follows: model 1, unilateral deposition (Riley & Weintraub, 1979; Seidman et al., 1979; Leffak, 1983); model 5, bilateral deposition (Russev & Tsanev, 1979); model 10, random deposition (Jackson & Chalkley, 1975a,b; Freedlander et al., 1977; Cusick et al., 1984). In these illustrations, the incorporation of IdUrd into DNA is indicated by dashed lines. The [^{3}H]lysine label of experiment 1 is denoted by (●), the [^{3}H]lysine label of experiment 2 by (○), and the [^{3}H]lysine label of experiment 3 by (■). (☆) Synthesis of histones for a generation prior to the labeling in generation 1 (experiment 1). The arrows indicate the daughter strand containing discontinuous DNA synthesis. N, normal density (no labeled histone on IdUrd-containing DNA); D, fully dense (all labeled histone on IdUrd-containing DNA); $^1/_2$D, half-dense (half the labeled histone on IdUrd-containing DNA).

al., 1978) that they can act as nucleation sites for the formation of nucleosomes and that H3–H3 interactions are maintained during several generations (Prior et al., 1980).

In Figure 8, we have illustrated 3 of the 11 mechanisms of histone deposition shown in Table I. We specifically illustrate these mechanisms as there is data in the literature in support of these specific models. Also shown is the expected distribution of labeled histone associated with IdUrd-containing DNA from the three experimental procedures described in Figure 1. Experimental procedure 2 requires in model 1 and 5 that the labeled histones remain associated with IdUrd-containing DNA after each replication event. The experimental data of Figure 4 clearly indicate that this is not the case. What is observed are the data illustrated in model 10. Further support for a random distribution has been reported by Cusick et al. (1984) using protocols similar to those originally used to support the unilateral deposition shown in Figure 8. Likewise, Freedlander et al. (1977) have reported procedures which indicate that 90% of the ^{3}H label in nuclear proteins segregates randomly with respect to DNA strands.

A potential source of ambiguity in these experimental procedures is the reincorporation of [^{3}H]lysine into histones during the chase periods which are from 20 to 40 h in length. The source of this [^{3}H]lysine would come from protein turnover. However, an extensive analysis of the level of reincorporation of radiolabel into histones indicates that reutilization amounts to only 4% for each chase period, a level vastly below that required to give the appearance of random distribution.

The data in this report indicate that parental histones segregate randomly with respect to the two daughter strands. This observation does not preclude an ordered mechanism with respect to other histones on the same daughter strands. For example, model 11 of Table I illustrates a random distribution with respect to which daughter strand parental (old) histones distribute. However, they may distribute in an ordered way with respect to the new histones of the type new–old–new–old or new–new–new–old–old–old, etc. (Pospelov et al., 1982; Annunziato & Seale, 1984). These procedures are not designed to answer that specific question. However, if such ordered deposition does exist, our data indicate that at the next replication event both ages of histone are distributed to both daughter strands on a random basis.

As discussed above, newly synthesized H2A and H2B probably interact with the bulk chromatin (or at least a specific part of it), thus providing them with an opportunity to exchange so that the H2A and H2B arriving in the region of the replication fork may consist of both old and new members as is observed experimentally. The question arises of why H2A and H2B should interact and exchange with similar histones in the bulk chromatin whereas new H3 and H4 do not, particularly since all nucleosomal core histones bind chromatin with great avidity in vitro. It seems highly likely that incoming H3 and H4 are associated with some factor which may weaken their ability to interact with chromatin but may permit binding to the more electrically negative DNA found immediately after the replication fork. Certainly newly deposited histones after the fork are unusually able to slide, perhaps reflecting a transient weaker histone–DNA binding (Jackson et al., 1981; Smith et al., 1984). Finally, we draw attention to the fact that a transient opportunity for a measure of nucleosome sliding immediately after replication could be of great value to the cell if it wished to establish new or reestablish old interactions between specific DNA sequence recognizing proteins and the DNA in the daughter chromosomes.

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Characterization of Phosphorylated Histidine-Containing Protein (HPr) of the Bacterial Phosphoenolpyruvate:Sugar Phosphotransferase System†

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ABSTRACT: The histidine-containing phosphocarrier protein (HPr) of the phosphoenolpyruvate:sugar phosphotransferase system, when phosphorylated, contains a 1-phosphohistidinyl (1-P-histidinyl) residue (His-15). The properties of this 1-P-histidinyl residue were investigated by using phospho-HPr (P-HPr), P-HPr-1, and P-HPr-2. HPr-1 and HPr-2 are deamidated forms of HPr produced by boiling. In addition, HPr-1 produced during frozen storage was investigated. Both pH and temperature dependencies of the rate of hydrolysis of the phosphoryl group of the 1-P-histidinyl residue were investigated. The results show that the 1-P-histidinyl residue in HPr and HPr-1 has significantly different properties from free 1-P-histidine and that these differences are attributable to the active-site residues Glu-66 and Arg-17 and the pK of the imidazole group of the 1-P-histidinyl residue in P-HPr. The 1-P-histidinyl residue in P-HPr and P-HPr-1 shows a greater lability at physiological pH than the free amino acid. A proposal for the active site of P-HPr is made on the basis of these results and the recently obtained tertiary structure. In contrast, the hydrolysis properties of the 1-P-histidinyl residue in P-HPr-2 were similar to those obtained for either free 1-P-histidine or denatured P-HPr. The loss of activity that is associated with boiling HPr was shown to be due to HPr-2 formation as HPr-1 was found to be fully active.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] in *Escherichia coli* and *Salmonella typhimurium* is responsible for the concomitant phosphorylation and translocation of a number of sugars. The PTS was initially described by Kundig et al. (1964) and has now been more fully described by Waygood et al. (1984). The sugar-specific phosphotransferases for glucose, mannose, fructose, and glucitol have been shown to be comprised of two enzymes and two phosphocarrier proteins as shown in Figure 1. The PTS specific for mannitol and *N*-acetylglucosamine do not have a factor III^{sugar} protein but do have a phosphorylated enzyme II^{sugar}. In addition, no factor III^{sugar} can be detected for the galactitol and dihydroxyacetone PTS (K. G. Peri and E. B. Waygood, unpublished results). Begley et al. (1982) concluded from stereochemical observations that there should be five phosphoryl transfer steps in the reaction mechanism of the glucose-specific PTS. This predicted the phosphorylation of

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[1] Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system(s); HPr, histidine-containing phosphocarrier proteins of the PTS; FPr, fructose-induced HPr-like phosphocarrier protein; HPr-1_{frozen}, HPr with one deamidation produced during frozen storage; HPr-1_{boil}, HPr with one deamidation produced by boiling; HPr-2, HPr with two deamidations produced by boiling; III^{sugar} or factor III^{sugar}, sugar-specific phosphocarrier protein of the PTS; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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