Nonrandom Distribution of Chromosomal Proteins during Cell Replication†

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ABSTRACT: The distribution of chromatin-associated proteins in replicating Chinese Hamster ovary cells has been examined using the method described by Taichman and Freedlender (Taichman, L., and Freedlender, E. F. (1976), *Biochemistry 15*, 447). Cells are grown for several generations in [^{14}C]lysine and thymidine, and then for one generation in the presence of [^{3}H]lysine and 5-bromodeoxyuridine (BrUdRib) and a further generation in cold amino acid and BrUdRib. This protocol produces equal amounts of unifilarly (heavy-light) and bifilarly (heavy-heavy) substituted DNA. Chromatin containing the two types of DNA are separated by sucrose-gradient centrifugation after ultraviolet irradiation. The results indicate that some of the chromatin proteins can segregate with the DNA strand synthesized in the same generation when the cells subsequently replicate. Using chromatin with a protein to DNA ratio of 2.6, in different experiments, 5–22% of the chromatin proteins were estimated to segregate with the appropriate DNA strand, while the remaining proteins were randomly distributed to daughter chromatin. The segregating proteins have not been specifically identified but they migrate in sodium dodecyl sulfate gel electrophoresis in the region where the four smaller histones migrate.

Many types of eukaryotic cells divide to produce daughter cells identical to the parent. Although the molecular systems controlling gene expression are maintained during replication, the details of this process are not understood. The possibility that control might be achieved by using a self-perpetuating arrangement of proteins on DNA was briefly considered by one of us some time ago (Smithies, 1970). Control proteins associated with DNA in a differentiated cell were postulated to direct the assembly of newly synthesized proteins onto newly replicated DNA to form DNA–protein complexes identical to those initially present. Tsanev and Sendov (1971) considered a similar situation in much greater detail when they postulated that the parental histones remain associated with the parental strand of DNA and direct the association of newly synthesized histones with the newly synthesized strand of DNA. Such nonrandom distribution, referred to as segregation, is the subject of this investigation.

The question of how parental chromosomal proteins are distributed to daughter chromatin has been asked previously but a clear case for either the random or the nonrandom mode has not been obtained. Prescott and Bender (1963) used autoradiography to follow the fate of pulse-labeled proteins in Chinese Hamster cells in tissue culture. They found little conservation of the labeled proteins; the small amount of label that was retained was equally distributed to both chromatids, indicating random association. More recently, Tsanev and Russev (1974) have obtained evidence in regenerating rat liver suggesting that some newly synthesized chromosomal proteins preferentially associate with newly synthesized DNA as predicted by the segregation model. While the proteins were referred to as histones, no attempt was made to characterize the labeled species and they were not followed through any succeeding cell divisions to determine whether they remained with the DNA strand. Jackson et al. (1975) followed the fate of radioactively labeled proteins and density-labeled DNA for several generations in tissue-cultured rat hepatoma cells. After correcting the lysine label for the contribution from nonhistone proteins, they concluded that histones are randomly distributed to the daughter DNA molecules. Their method would probably not have been able to detect a small percentage of segregating proteins.

We describe here experiments designed to examine directly the distribution of chromosomal proteins after one and two rounds of DNA replication. The method of Taichman and Freedlender (1976) separates unfixed chromatin-containing DNA substituted with bromodeoxyuridine (BrUdRib)[1] in one strand of the DNA (HL chromatin) from chromatin with both strands of the DNA substituted (HH chromatin). Irradiation at 313 nm causes HH chromatin to break into smaller fragments than HL chromatin; the two species may then be partially separated on sucrose gradients. This technique makes it possible to follow both the proteins and the DNA synthesized in a given generation by radioactively labeling the proteins and substituting the DNA with BrUdRib. The results obtained from many experiments using a chromatin with a protein to DNA ratio of 2.6 indicate that during cell replication 5–22% of the chromatin proteins remain associated with the DNA strand synthesized in the same generation; the remainder of the proteins are randomly distributed to daughter chromatin.

Experimental Design

In order to make our experiments easier to follow, we present a typical labeling protocol and the theoretical outcomes predicted by the segregation and random models in Figure 1. Cells are first grown in the presence of thymidine and ^{14}C-labeled

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[1] Abbreviations used are: BrUdRib, 5-bromodeoxyuridine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CHO, Chinese Hamster ovary cells; EDTA, (ethylenedinitrilo)tetraacetic acid.

amino acid; then they are grown for one generation in the presence of BrUdRib and ^{3}H-labeled amino acid; this is followed by one further generation in BrUdRib and unlabeled amino acid. The results expected under the two models are quite different.

The segregation model predicts that ^{14}C-labeled proteins synthesized in the first generation along with light (L) DNA will remain associated with this L strand. Likewise, the ^{3}H-labeled proteins synthesized in the second generation along with a heavy (H) strand of DNA will remain with it. After a third generation, producing unlabeled proteins and a heavy strand of DNA, the ^{14}C-labeled proteins will be associated with HL DNA, while the ^{3}H-labeled proteins are associated with HH DNA. After irradiation, the HH chromatin fragments bearing ^{3}H-labeled proteins should be smaller and sediment less rapidly in sucrose gradients than the ^{14}C-labeled HL fragments. Thus, the ^{3}H/^{14}C ratio should increase towards the top of the gradient. For proteins which are randomly distributed during replication, both labels will be equally associated with the two types of DNA. Thus, the ^{3}H/^{14}C ratio should be constant across the gradient.

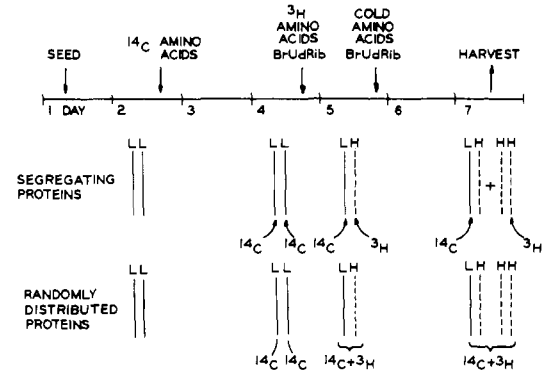

FIGURE 1: Schematic representation of the labeling protocol for CHO cells and the expected distribution of the label for segregating and randomly distributed proteins. The cells were cultured and labeled as described under Methods. The DNA is represented in the lower portion of the figure by two vertical lines: a thymidine-containing (L) strand of DNA is indicated by a solid line and a BrUdRib-substituted (H) strand by a dashed line. The ^{14}C and ^{3}H refer to proteins labeled with these isotopes. The arrows denote the strand with which these labeled proteins are complexed.

Materials and Methods

Incorporation of Radioactive Tracers and BrUdRib. Chinese Hamster ovary cells (CHO) were maintained in F10 medium (Ham, 1963) containing 15% calf serum (Gibco), 7.5 mM Hepes buffer (Calbiochem), 100 units/mL penicillin and 100 μg/mL streptomycin. The doubling time was 19 h. Cells were first labeled with ^{14}C-labeled amino acid by growing for 2 days in medium containing one-half the normal level of cold amino acid (F10-AA/2) and 1 μCi/mL ^{14}C-labeled amino acid. The cells were then incubated for 1 h in F10-AA/2 and labeled for 20 h with 6 μCi/mL ^{3}H-labeled amino acid in F10-AA/2 and 1.5×10^{-4} M bromodeoxyuridine (BrUdRib). Following a 1-h rinse in fresh F10 and 1.5×10^{-4} M BrUdRib, the cells were grown for 3 additional days in the same medium and harvested. The amino acids used for labeling were [^{14}C]lysine (287 mCi/mmol) or [^{14}C]leucine (324 mCi/mmol) followed by [^{3}H]lysine (18 Ci/mol) or [^{3}H]leucine (57 Ci/mmol). In some cases, the order of labeling was reversed, with the ^{3}H-labeled amino acid being incorporated first, followed by the ^{14}C-labeled amino acid.

For mixing experiments, ^{14}C-labeled HL chromatin was prepared from cells grown for one generation in the presence of [^{14}C]lysine (1/μCi/mL) and 1.5×10^{-4} M BrUdRib. To obtain ^{3}H-labeled HH and HL chromatin, cells were grown for one generation in the presence of [^{3}H]lysine and 1.5×10^{-4} M BrUdRib, followed by a generation of cold lysine and BrUdRib.

Isolation and Separation of HL and HH Chromatin. Chromatin was isolated from cells as described by Taichman and Freedlender (1976). This chromatin has a protein to DNA ratio of 2.6. The sheared chromatin in 0.1 mM EDTA, pH 8.0, was irradiated for 18 h in a quartz cuvette placed in an aluminum jacket, cooled to 0 °C by a Forma refrigerant bath. To avoid troublesome condensation problems, the entire apparatus was placed in a cold room. The cuvette was placed 3 cm from a General Electric H 100 A 4/T mercury lamp behind a composite filter made from one Corning No. 7058 glass filter and one Corning No. 7740 glass filter between which was 3 mm of a circulating aqueous solution of 200 mg of K_2CrO_4/L. This filter has a peak transmission at 313 nm and a half-height bandwidth of about 20 nm. Light transmission is less than 1% below 295 nm and above 350 nm. After irradiation, the chromatin was made 0.015 M NaCl and 0.0015 M sodium citrate, pH 7.0, incubated for 10 min at 37 °C, and centrifuged on a 9–30% sucrose gradient as described by Taichman and Freedlender (1976). Aliquots (0.1 mL) of each gradient fraction were counted for radioactivity in Bray's (1960) solution. Appropriate quench corrections were applied using standard curves made with chromatin containing only a single label.

Sodium Dodecyl Sulfate Gel Electrophoresis of Labeled Chromatin-Associated Proteins. To examine the chromatin-associated proteins, sucrose gradient fractions were pooled, mixed with unlabeled chromatin (1 OD_{260} unit), dissolved in sample buffer, and electrophoresed as described by Laemmli (1970) in 12% (w/v) acrylamide gels using 1.28% (w/v) *N,N'*-diallyltartardiamide (Bio-Rad) as a cross-linker. Gel fractions (2 mm) were obtained with a Gilson gel fractionator, hydrolyzed with 1 mL of 2% (w/v) periodic acid, and counted in a Triton-xylene scintillation fluid (Anderson and McClure, 1973).

Results

Validation of Method for Protein Labeled Chromatin. Taichman and Freedlender (1976) have shown that HL chromatin labeled with [^{14}C]thymidine can be partially resolved from HH chromatin labeled with [^{3}H]BrUdRib by sedimentation in sucrose gradients after irradiation at 313 nm. The HL chromatin is not as extensively fragmented as the HH chromatin and therefore sediments farther into the gradient. In order to determine whether the separation of the two types of chromatin can be followed if the label is in the chromatin-associated proteins, a mixing experiment was performed. Nuclei containing only [^{14}C]lysine-labeled HL chromatin were mixed with nuclei containing [^{3}H]lysine-labeled HH and HL chromatin. The chromatin was isolated from the mixed nuclei, irradiated, and centrifuged on a 9–30% sucrose gradient as described under Materials and Methods. Figure 2 shows the distribution of radioactive labels in such an experiment. The ^{14}C label which is only in HL chromatin sediments farther into the gradient than the ^{3}H label which is in HH and HL chromatin. The relative enrichment of ^{14}C near the bottom of the gradient and of ^{3}H near the top is reflected in the pattern of the log (^{3}H/^{14}C) ratio (Figure 2, top) which is higher at the top of the gradient. (The log of the ^{3}H/^{14}C ratio is used so that a twofold change from 1 to 2, for example, will have the same weight as a twofold change from 0.5 to 1.0.)

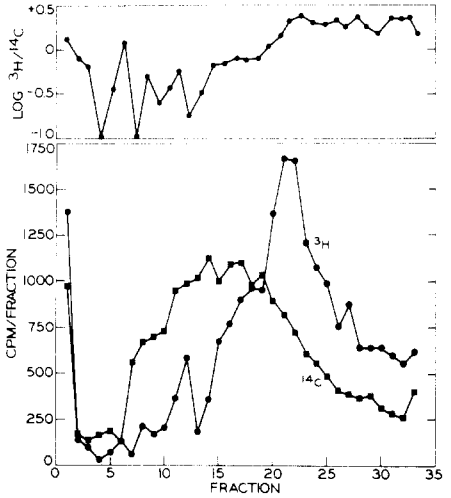

FIGURE 2: Sucrose gradient sedimentation of a reconstituted mixture of protein-labeled chromatins. Nuclei containing [^{14}C]lysine-labeled HL chromatin were mixed with nuclei containing [^{3}H]lysine-labeled HH chromatin. After isolation and irradiation, the chromatin was sedimented in a 9–30% sucrose gradient. Lower panel: (●) ^{3}H cpm; (■) ^{14}C cpm. Upper panel: log (^{3}H/^{14}C) ratio.

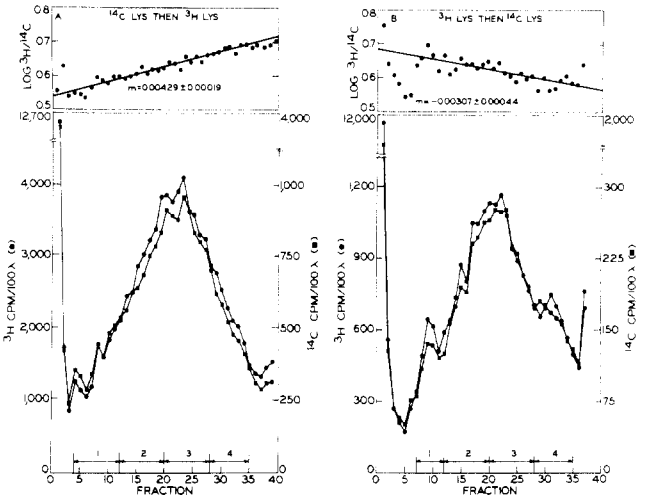

FIGURE 3: Sucrose gradient sedimentation of lysine-labeled chromatin. (A) Cells were grown first in [^{14}C]lysine and thymidine medium followed by one generation in [^{3}H]lysine and BrUdRib and a further generation in cold lysine and BrUdRib. Chromatin was isolated, irradiated, and centrifuged on a 9–30% sucrose gradient. (B) Same as A, except that the order of labeling was reversed. Cells were grown first in [^{3}H]lysine and thymidine followed by [^{14}C]lysine and BrUdRib. Lower panels: (■) ^{14}C cpm; (●) ^{3}H cpm. The numbers along the bottom refer to pools made for gel analysis of the proteins. Upper panels: in the upper panels the log (^{3}H/^{14}C) ratio is indicated by a dot (•) while the line represents the calculated least-squares regression line through the points.

In this particular experiment, the low levels of absolute counts at the bottom of the gradient produce a high amount of scatter in the ^{3}H/^{14}C ratios in this region. The mixing experiment has, however, been repeated five times and in every case the ^{3}H/^{14}C ratio of the rapidly sedimenting chromatin has been significantly different from that of the more slowly sedimenting material. These experiments establish that the separation of HL and HH chromatin can be observed using protein-labeled chromatin. It further indicates that the conditions of chromatin preparation do not cause a large redistribution of chromosomal proteins, at least after the stage of isolating the nuclei. The same result is obtained even after prolonged incubation of the chromatin gel in the nuclear isolation buffer. Thus, it appears that, if any redistribution of protein occurs during the complete preparative procedures, it is not sufficient to make the method unsuitable for studying the distribution of chromosomal proteins during cell replication.

Sucrose Gradient Centrifugation of Lysine-Labeled Chromatin. Cells were labeled first with [^{14}C]lysine in thymidine-containing medium, then for one generation with [^{3}H]lysine in the presence of BrUdRib and grown for further generation with unlabeled lysine and BrUdRib (see Figure 1). Analytical CsCl density gradient centrifugation of DNA isolated from these cells showed that half of the DNA has a density of 1.748 g/mL (that of fully substituted HL DNA) while the rest has a density of 1.797 g/mL (that of HH DNA). Thus, the DNA has replicated twice in the presence of BrUdRib as expected.

In order to test the hypothesis that some chromosomal proteins segregate, it is essential to establish that no protein label is incorporated into DNA producing artifactual "protein" segregation. To this end, chromatin labeled with lysine according to the protocol in Figure 1 was examined in $CsSO_4$ gradients. As expected for protein, but not DNA, both labels remained at the top of the gradient. RNase and DNase digestions before centrifugation did not affect the pattern. Pronase digestion, on the other hand, left only background levels of counts throughout the gradient, as would be expected if the labels are only in protein. Similar pronase treatment of chromatin labeled with [^{3}H]BrUdRib and [^{14}C]thymidine resulted in the pattern expected from DNA, i.e., two clearly separated peaks corresponding to HH [^{3}H]DNA and HL [^{14}C]DNA. There was a 100-fold change in the ^{3}H/^{14}C ratio between the HH and HL DNA peaks. In the case of the lysine-labeled material, no change in the ^{3}H/^{14}C ratio could be detected across the gradient, including the regions where HH and HL DNA would be banded. These various experiments establish that lysine does not enter the DNA to any detectable extent. Cells labeled according to the protocol in Figure 1 are therefore suitable for studying the segregation of chromosomal proteins without any complications from DNA segregation.

Segregation of these proteins was examined by sedimenting irradiated chromatin in a 9–30% sucrose gradient. The results of an experiment of this type are shown in Figure 3A. The labeled material sediments in a broad band with 65% of the label remaining in the gradient. The ^{14}C and ^{3}H counts are not superimposable; there is an enrichment of ^{14}C label in the faster-sedimenting material and ^{3}H label towards the top of the gradient. This is more clearly seen by examining the log (^{3}H/^{14}C) ratio across the gradient (Figure 3A, top). Linear regression analysis of the log (^{3}H/^{14}C) ratio vs. fraction number indicates that there is a statistically significant ($p < 0.01$) rise in the ratio from the bottom to top of the gradient. These results indicate that there is an enrichment of ^{14}C-labeled proteins with the HL DNA.

If the results are not artifactual, reversing the order of labeling should produce an enrichment of ^{3}H label towards the bottom of the gradient and ^{14}C material towards the top. Figure 3B presents the results of an experiment with the labels reversed. As expected, there is now an enrichment of ^{3}H label in the HL region towards the bottom of the gradient and more ^{14}C material in the HH region towards the top. This is reflected in the decrease of the log (^{3}H/^{14}C) ratio towards the top of the gradient. Again, the linear regression line has a statistically significant ($p < 0.01$) slope. The slopes of the log (^{3}H/^{14}C) ratio with the two types of labeling have the signs expected if some proteins remain associated with the DNA synthesized

TABLE I: Summary of Sucrose Gradients.

	Culture	Irradiation	Gradient	Slope	SD(±)[a]	p	% Segregation
	1	A	1	5.95	1.30	<0.01	22.5
		B	2	2.08	0.473	<0.01	7.5
[^{14}C]Lysine	2	C	3	3.66	0.98	<0.01	13.0
followed by	3	D	4	4.29	0.19	<0.01	16.0
[^{3}H]lysine	4	E	5	0.18	0.37	0.65	NS[b]
		F	6	0.18	0.64	0.8	NS
		G	7	2.31	0.42	<0.01	8.2
		H	8	1.49	0.50	<0.01	5.0
[^{14}C]Leucine	5	I	9	−0.87	0.48	0.1	NS
followed by		J	10	2.09	0.40	<0.01	7.5
[^{3}H]leucine		J	11	0.88	0.41	0.04	NS
	6	K	12	−3.02	0.42	<0.01	11.0
		L	13	−4.47	0.49	<0.01	16.7
	7	M	14	−0.75	0.78	0.35	NS
[^{3}H]Lysine		M	15	−2.92	0.79	<0.01	10.7
followed by		N	16	−1.00	0.88	0.25	NS
[^{14}C]lysine		N	17	−3.07	0.44	<0.01	11.2
	8	O	18	0.82	1.29	0.5	NS
	9	P	19	−1.92	0.49	<0.01	6.8
		P	20	−0.59	0.65	0.35	NS
[^{3}H]Leucine	10	Q	21	−3.48	0.52	<0.01	12.7
followed by		Q	22	−3.32	0.49	<0.01	12.2
[^{14}C]leucine						Av.	11.5 ± 4.6
Theoretical				1.37			5
(from Figure				2.74			10
5B)				4.11			15
				5.29			20

[a] Standard deviation. [b] Not statistically significant.

in the same generation. The fact that the slope of the log (^{3}H/^{14}C) line reverses when the order of labeling is reversed rules out a number of artifacts, including the possibility that the results shown in Figure 3A might be caused by quench correction error or by a preferential loss of ^{3}H compared to ^{14}C during the ultraviolet irradiation. (We found, in other experiments, that generally labeled [^{3}H]tryptophan lost ^{3}H label during irradiation.)

Although the separation of the two labels on the gradients appears small, the results are reproducible. Table I summarizes the data from 22 separate gradients using material from ten independent cell labelings. Both lysine and leucine have been used as protein labels. A statistically significant ($p < 0.01$) slope for the log (^{3}H/^{14}C) ratio has been obtained in 14 gradients representing all but one of the labelings, and in each case the slope has had the sign predicted from the order of labeling. Statistically significant slopes were not obtained in eight cases. One labeling gave no significant slope, presumably because the cells failed to go through two rounds of replication in BrUdRib. The other failures are probably due to technical difficulties with the gradients as indicated by the wide scatter of the data in these particular experiments.

Sodium Dodecyl Sulfate Gel Electrophoresis of Proteins Isolated from Gradient Fractions. In order to ensure that it is the protein component of chromatin which is segregating and to characterize this species further, the proteins were isolated from pooled gradient fractions and examined by sodium dodecyl sulfate gel electrophoresis. The graph shown in Figure 4 represents the pattern of radioactivity obtained after electrophoresis of the proteins from one pool of a typical sucrose gradient. For comparison, the staining pattern of a preparation of unirradiated chromosomal proteins electrophoresed in the same system is shown at the bottom of the figure. The electrophoresis patterns obtained with the proteins from different pools from the same gradient were essentially indistinguishable, except in their ^{3}H/^{14}C ratios. A large proportion of the labeled protein migrates rapidly in the area of the gel (75–95 mm) where histones H2A, H2B, H3, and H4 migrate. Control

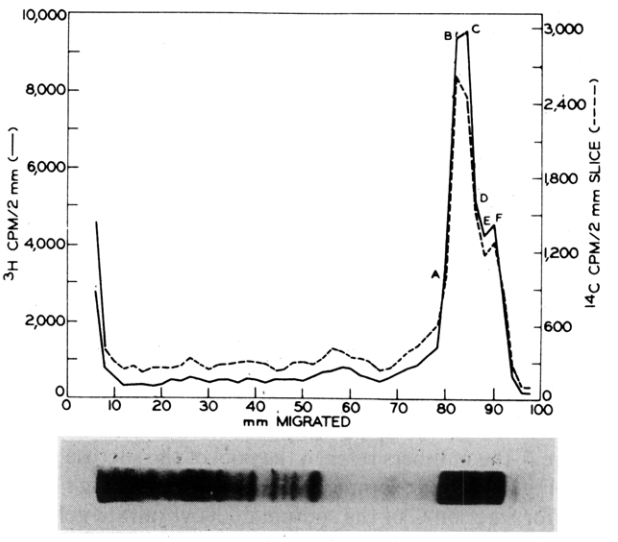

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of proteins isolated from pooled gradient fractions. The proteins were isolated from gradient fractions pooled as indicated in Figure 3A and electrophoresed in 12% sodium dodecyl sulfate gels as described under Methods. Gels were crushed and counted. (—) ^{3}H cpm; (- - -) ^{14}C cpm. The letters refer to the fractions for which the log (^{3}H/^{14}C) ratios are plotted in Figure 5. Below: A typical gel of total nuclear proteins stained with Coomassie blue.

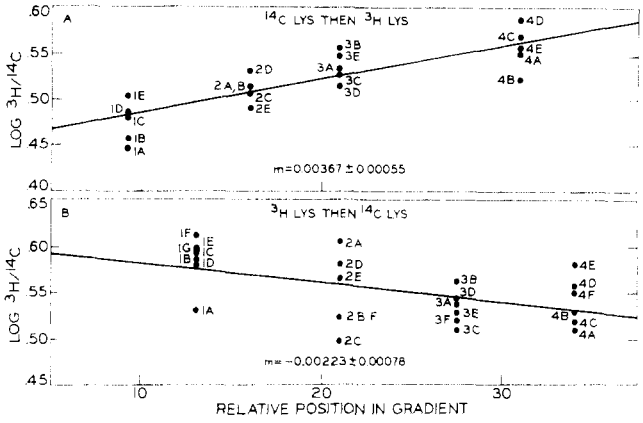

FIGURE 5: Log (^{3}H/^{14}C) ratios in proteins from different fractions of the sucrose gradients. The proteins from the sucrose gradient fractions, pooled as in Figure 3, were analyzed by sodium dodecyl sulfate gel electrophoresis (Figure 4). The log (^{3}H/^{14}C) ratios of the gel fractions in the major peak were plotted against the mean sucrose gradient fraction from which the material originated. These ratios are indicated by the points (•). The numbers refer to the pool (Figure 3) and the letters to the gel fraction (Figure 4). The line is the calculated least-squares regression line. Lower: Log (^{3}H/^{14}C) ratios from gels of gradient fractions from Figure 3b. Cells were labeled first with [^{3}H]lysine, followed by [^{14}C]lysine. Upper: Log (^{3}H/^{14}C) ratios from gels of gradient fractions from Figure 3A. Cells were labeled first with [^{14}C]lysine, followed by [^{3}H]lysine.

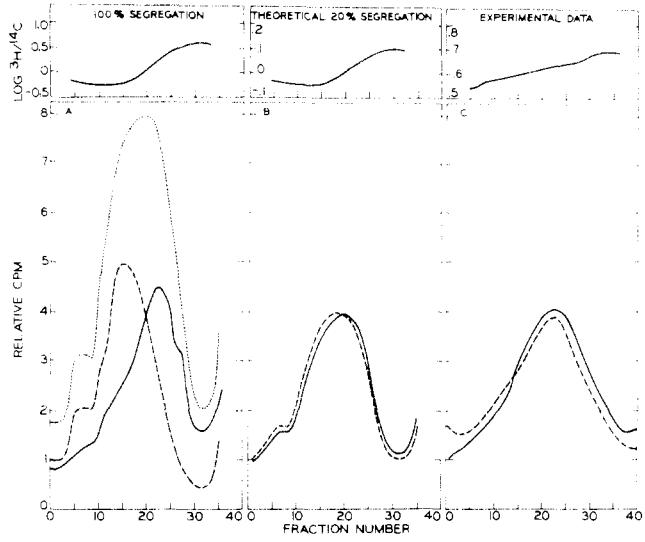

FIGURE 6: Method of calculating the percentage of segregating proteins. Upper: Log (^{3}H/^{14}C) ratios. Lower: Counts per minute. (A) Experimental data obtained with chromatin labeled in the DNA. This pattern is the same as would be obtained with 100% segregating proteins. (- - -) [^{14}C]Thymidine-labeled HL chromatin; (—) [^{3}H]BrUdRib-labeled HH chromatin; (• • •) sum of ^{14}C and ^{3}H curves. (B) Calculated curves expected for 20% segregation. This represents the pattern which would be obtained with chromatin labeled first with [^{14}C]lysine followed by [^{3}H]lysine if 20% of the labeled proteins segregated. (- - -) ^{14}C; (—) ^{3}H. (C) Experimental data from Figure 3A. (- - -) ^{14}C; (—) ^{3}H.

experiments with tryptophan-labeled unirradiated chromatin show that only a small amount of the nonhistone proteins also migrate to this position. The region of the gel where histone H1 should appear does not contain much label. This suggests a loss of H1 during the experimental manipulations and means that these experiments give no information on the mode of distribution of this histone.

The ^{3}H/^{14}C ratios of the proteins associated with chromatin from different parts of the sucrose gradients were compared in the following manner. First, the fractions from a gradient were combined into four pools as indicated at the bottom of Figure 3; these were numbered one through four. Proteins were isolated from each pool and electrophoresed on separate gels. The logs of the ^{3}H/^{14}C ratio of the gel fractions across the major peak of the sodium dodecyl sulfate gel (indicated by the letters in Figure 4) were plotted against the pool position in the sucrose gradient from which the proteins were isolated (Figure 5); the position of the pool in the sucrose gradient is plotted on the horizontal axis according to its mean fraction number. The individual points plotted are the log of the ^{3}H/^{14}C ratios for the sodium dodecyl sulfate gel fractions lettered as shown in Figure 4; the numbers refer to the pool of the sucrose gradient as in Figure 3. For example, point 3C shows the log (^{3}H/^{14}C) ratio for fraction C of the sodium dodecyl sulfate gel electrophoresis of proteins from sucrose gradient pool 3.

The ^{3}H/^{14}C ratios across the peak of an sodium dodecyl sulfate gel show no systematic changes; i.e., in any one gel there is no reproducible systematic change in ^{3}H/^{14}C ratio in going from gel fractions A to E. The ratios, however, do differ between different gradient pools; i.e., there is a systematic change in ^{3}H/^{14}C ratio in going from gradient pool 1 to 4. This is illustrated by the straight lines drawn through the data points; these lines were determined by least-squares regression analysis and, in both cases, have statistically significant slopes ($p < 0.01$). No single gel fraction appears to contribute disproportionately to the overall slopes.

When cells are labeled first with ^{3}H-labeled amino acid and then with ^{14}C-labeled amino acid, the segregation model (see Figure 1) predicts an enrichment of ^{3}H-labeled proteins in HL material near the bottom of the gradient and ^{14}C-labeled proteins in HH material towards the top and a negative slope for the ^{3}H/^{14}C regression line. When the order of labeling is reversed, i.e., ^{14}C-labeled amino acid first followed by ^{3}H-labeled amino acid, a positive slope should be obtained. These predictions are borne out by the sodium dodecyl sulfate gel data.

Control gels with chromatin labeled in the DNA show no label in the running gel. Controls in which the chromatin was digested with DNase prior to electrophoresis produce the same pattern as shown here. This result was expected, since our $CsSO_4$ gradient control experiments reported above showed no protein label in the DNA. The sodium dodecyl sulfate gel analysis therefore confirms the data obtained directly from the sucrose gradients and demonstrates that it is the protein components of the chromatin which are responsible for the change in the ^{3}H/^{14}C ratio. The gels also suggest that the major proteins concerned migrate in sodium dodecyl sulfate gels in the same region as the four smaller histones. The data do not allow us to say whether the segregating species is one or a pair of the four smaller histones, or some non-histones, or a small percentage of all the histones. There are two technical difficulties which have prevented identification of the segregating species. Irradiation slightly alters the electrophoretic mobilities of some proteins in the sodium dodecyl sulfate gels, and, after concentrating the sucrose gradient pools, we have been unable to resolubilize enough counts in urea to use the Panyim and Chalkley (1969) gel system for histones.

Estimation of the Percentage of Segregating Proteins. The separation of the ^{3}H and ^{14}C labels on the sucrose gradients appears slight, suggesting that only part of the chromatin proteins segregate in a nonrandom fashion, while the remainder are randomly distributed. The percentage which segregate nonrandomly may be estimated as follows. The sedimentation pattern of chromatin containing [^{14}C]thymi-

dine-labeled HL DNA and [^{3}H]BrUdRib-labeled HH DNA is equivalent to that expected in the case where 100% of the proteins remain with the DNA synthesized in the same generation. The sum of these ^{14}C and ^{3}H curves is the pattern expected in the case where all proteins are randomly distributed. The ^{14}C and ^{3}H curves in the lower part of Figure 6A are normalized data from a control experiment in which HL chromatin was labeled with [^{14}C]thymidine and HH chromatin with [^{3}H]BrUdRib. The theoretical curves expected for various percentages of segregating and randomly distributed proteins may be calculated by summing the appropriately weighted curves of Figure 6A. The pattern expected for the case in which 20% of the proteins segregate is shown in the lower part of Figure 6B. This expected result can be compared with an actual experiment shown in the lower part of Figure 6C (data from Figure 3A). The two are very similar except that the experimental log (^{3}H/^{14}C) curve does not show the pronounced S shape seen in the theoretical curve. The S shape is always visible in curves from mixing experiments and experiments with DNA-labeled material but was only observed in 3 of the 22 gradients with protein-labeled material. We do not understand the reason for this difference.

The logarithms of the ^{3}H/^{14}C ratios were used to estimate quantitatively the percentage of segregating protein. (The logarithms are used so that a twofold change from 0.5 to 1 has the same weight as a change from 1 to 2.) When least-squares straight lines are fitted to the data, the magnitude of the slope of this line in the reconstruction experiment is proportional to the assumed percentage of segregating proteins. Thus, we can use the observed slope from this type of analysis to make a rough estimate of the percentage of proteins which segregate. Table I compares the slope expected for various percentages (calculated using this procedure) with the actual values obtained from the sucrose gradient analyses. These data indicate that between roughly 5 and 22% of the labeled proteins are segregating. Values in the same range have also been obtained in the sodium dodecyl sulfate gel analyses. We have no means at present of checking the correctness of these values, and regard them only as an estimate of the order of magnitude.

Discussion

These experiments were designed to test the hypothesis that some newly synthesized chromosomal proteins remain associated with DNA synthesized in the same generation. The results described indicate that an appreciable proportion of the total chromosomal proteins is distributed to daughter chromatin as suggested by the hypothesis. The amount of segregating material was roughly estimated to be between 5 and 22% of the labeled proteins.

Several factors could contribute to randomization of the labels during the course of the experiment. Although the shearing of the chromatin prior to irradiation is mild, it may cause exchanges of protein in the chromatin gel which could randomize the label. Any other causes of exchange of proteins during the isolation would also tend to randomize the labels. Exchange of proteins after the stage of lysing the nuclei is probably not a significant cause of label randomization, as shown by the mixing experiments in which nuclei containing [^{14}C]lysine-labeled HL material were mixed with nuclei containing [^{3}H]lysine-labeled HH chromatin. The separation of the two labels on sucrose gradients was similar to that obtained when the chromatin is labeled in DNA. Similarly, the experiments with DNA-labeled material rule out significant randomization by sister chromatid exchange during mitosis. The percentage of segregating proteins detected has varied from 5 to 22%; the average of all the statistically significant gradients is 11%. Since the expected experimental artifacts tend to decrease the amount of segregation observed, the actual value may be closer to 20%.

The largest source of potential bias in this study relates to the use of BrUdRib to label the DNA strands. There is evidence that proteins have a higher affinity for BrUdRib-substituted DNA than for thymidine-containing DNA (Lin et al., 1976; Gordon et al., 1976). It is difficult to predict how this might affect our results. If first-generation proteins are normally preferentially associated with the first-generation strand of the DNA and second-generation proteins with the second-generation strand, this association might be easier to detect as a result of an increased affinity, although the differential association would not have been caused by the BrUdRib. If first- and second-generation proteins are normally associated equally with the second-generation DNA strand, then an increased affinity would not create a bias. On the other hand, preferential binding of proteins to the BrUdRib-substituted strand could cause some first-generation proteins to go with the second-generation (H) strand when ordinarily they would stay with the first-generation (L) strand. This would increase the amount of first label in the HH material and decrease the amount of segregation observed. Although we cannot rigorously exclude a bias in our results caused by the use of BrUdRib, we are unable to see how it could create artifactual segregation.

Some of the segregating proteins comigrate with the four smaller histones in sodium dodecyl sulfate gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis of control unirradiated chromatin preparations indicates that about 40% of the lysine label is in these smaller histones. Since less than 40% of the labeled material segregates, many of the histones must be randomly distributed. The data are compatible with the possibility that one or a pair of the four smaller histone species (H2A, H2B, H3, or H4) is segregating. The data are also compatible with the possibility that a fraction of all the histones segregate while others do not. Small nonhistone proteins might also be candidates for the segregating species. In the present work, we deliberately used chromatin with a high protein to DNA ratio so that we would not miss segregating components which might be removed during purification. Future experiments with more highly purified chromatin should help in identifying the segregating species more precisely.

The observations that histones can reassociate with bacterial and viral DNA as well as eukaryotic DNA to produce a characteristic subunit structure indicate that the maintenance of histone–DNA complexes during replication is not necessary for the formation of these structures. This does not, however, eliminate the possibility that subunits in vivo occur at specific sites or have arrangements which are nonrandom. For example, the arrangement symbolized as H3:H4 might be different from that symbolized as H4:H3. This difference and/or the precise location of the subunit on the DNA could be preserved by a self-directing assembly mechanism.

Self-directing assembly could equally well provide the means by which the complexes of control proteins with DNA, first established during "determination", are faithfully reproduced during replication of the determined cell. The finding that some chromosomal proteins do remain associated with the DNA strand synthesized in the same generation suggests that such proteins may be important in the faithful reproduction of the protein–DNA complexes during cell replication and may thus be involved in the maintenance of the differentiated state. If this is true, an alteration in the normal pattern of regulatory protein–DNA complexes might also be stably transmitted to

subsequent generations producing a stable phenotypic but not genotypic change in the cell population. Further experiments are underway to identify the nature and function of the newly synthesized chromosomal proteins which have been shown here to remain associated with the DNA strand synthesized in the same cell cycle.

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Deoxyribonucleic Acid Chain Growth and Organization of Replicating Units in HeLa Cells†

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ABSTRACT: A method for studying DNA chain growth and chromosomal organization of replicons in HeLa cells has been developed. DNA replication is initiated with bromodeoxyuridine followed by pulse labeling of active replicons with [^{3}H]thymidine and growth of the chains for finite intervals in unlabeled thymidine. Photolysis of the bromodeoxyuridine-DNA leader with 313-nm light releases the newly replicated chains that are then analyzed by sedimentation in alkaline sucrose gradients. This method of analysis provides data on the rate of chain growth, the bidirectionality of replication, and the distribution of the active replicons at specific intervals in the S period. Applying this method to cells caused to synthesize DNA at a lowered temperature (27 °C) or with protein synthesis restricted by cycloheximide revealed that the immediate reduction in the rate of DNA replication in both instances was due to a decreased rate of chain growth without derangement of the overall process.

DNA replication in the nucleus of eukaryotic cells involves the temporally ordered replication of many subchromosomal DNA units (replicons) which vary from 20 to 70 μm in length (Cairns, 1966; Huberman and Riggs, 1968; Callan, 1972). The ordering of the initiation of DNA synthesis in specific replicons remains unexplained; however, active replicons tend to be located in clusters giving rise to distinctive labeling patterns in different chromosomes (Taylor, 1959; Stubblefield and Mueller, 1962; Huberman and Riggs, 1968; Hori and Lark, 1973; Hand, 1975a). When a particular replicon is activated, the synthesis of DNA usually begins in the middle of the unit and proceeds in both directions (Huberman and Riggs, 1968; Huberman and Tsai, 1973; Weintraub, 1972; Hand and Tamm, 1973). The chain growth from this point in the overall 5′ → 3′ direction appears to proceed by the discontinuous formation of 5-6S pieces (Okazaki fragments) that are ligated to form mature DNA (Schandl and Taylor, 1969; Nuzzo et al., 1970; Kidwell and Mueller, 1969; Goldstein and Rutman, 1973; Huberman and Horwitz, 1973; Gautschi and Clarkson, 1975). The opposing strand may proceed by the same mechanism or by direct extension (Hershey and Taylor, 1974; Friedman, 1974). The molecular events which regulate the initiation of specific segments, the chain growth rates, and ligation of adjacent replicons are as yet little understood.

The present study is directed to the development of a method for investigating DNA chain growth and the organization of replicating units during chromosomal replication both in living cells and in subcellular systems. The general protocol involves the initiation of DNA replication in synchronized cells with bromodeoxyuridine so as to introduce a photolabile DNA segment in each active replicon, a subsequent pulse labeling

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