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Separation of Chromatin Containing Bromodeoxyuridine in One or Both Strands of the DNA†

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ABSTRACT: A method has been devised for the separation of chromatin containing 5-bromodeoxyuridine (BrUdRib) in one strand (HL) of the DNA from that with BrUdRib in both strands (HH). Ultraviolet light breaks chromatin containing HH DNA into smaller fragments than chromatin containing HL DNA and the two species can be partially resolved on neutral sucrose gradients. Unfiltered ultraviolet light is not suitable since it causes considerable alteration in the electrophoretic pattern of chromatin-associated proteins. Irradiation with 313-nm light causes much less damage to the associated proteins. The ability to separate, isolate, and examine chromatin containing HL and HH DNA makes studies on the distribution of chromatin-associated proteins possible.

The replication of eukaryotic chromatin involves the semiconservative synthesis of DNA (Taylor et al., 1957; Chun and Littlefield, 1961) and the association of chromosomal proteins with DNA in a specific manner to yield daughter molecules that are functionally identical with the parent. How the proteins present in parental chromatin are distributed to the daughter chromatin has for sometime been a matter of interest (Prescott and Bender, 1963: Weintraub, 1973; Tsanev and Russev, 1974). This communication details a method for separating chromatin containing DNA with BrUdRib[1] in one strand from chromatin containing DNA with both strands substituted. The technique should facilitate investigations of the distribution of chromatin-associated proteins during cell replication.

The technique is based on the observation that irradiation of DNA containing BrUdRib in both strands causes double-stranded breaks (Hutchinson, 1973). The primary lesion induced by the ultraviolet light is a single-strand nick at the site of a photoactivated BrUdRib molecule (Hutchinson, 1973). Double-stranded breaks occur where two single-strand nicks are sufficiently close together in opposite strands (Freifelder and Trumbo, 1969). Fewer double-strand breaks would be induced in DNA containing BrUdRib in one strand (HL DNA) than in DNA containing BrUdRib in both strands (HH DNA) because the unsubstituted (L) strand in the HL molecule breaks with much lower frequency than the substituted (H) strand (Hutchinson, 1973). Such differential sensitivity of HL and HH DNA suggests that ultraviolet light might also cause chromatin containing HH DNA to break into smaller fragments than chromatin containing HL DNA. The present communication describes experiments which demonstrate the validity of this prediction and detail a procedure for separating the two species of chromatin. In addition a method is given for extraction and gel electrophoresis of chromatin-associated proteins from the resultant products.

Methods

Cells and Culture Media. Chinese Hamster Ovary (CHO) cells (Puck et al., 1958) were cultured on a roller apparatus in F10 medium (Ham, 1963) containing 15% calf serum (Gibco), 7.5 m*M* Hepes buffer (Calbiochem), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The doubling time was 19 hr. "F10 minus Thy" medium is F10 medium lacking thymidine.

Incorporation of BrUdRib and Radioactive Tracers. To

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[1] Abbreviations used are: BrUdRib, 5-bromodeoxyuridine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

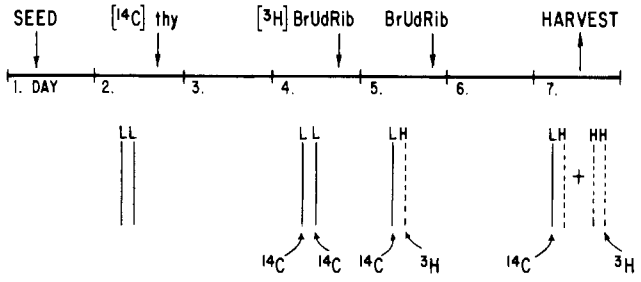

FIGURE 1: Schematic representation of labeling protocol for CHO cells. The cells were cultured and labeled as described in Methods. The DNA is represented in the lower portion of the figure by two vertical lines: an unsubstituted strand (L) of DNA is indicated by a solid line and a BrUdRib substituted strand (H) by a dotted line. The location of the ^{14}C and ^{3}H isotopes are indicated by arrows to the appropriate strand. Although not shown, on day 4 some molecules of DNA are unlabeled in one strand; this unlabeled light strand does not alter the results nor their interpretation.

label HL chromatin with [^{14}C]thymidine and HH chromatin with [^{3}H]BrUdRib (Figure 1), 1.5×10^6 cells were seeded in a 16-oz. amber bottle. On day 2, the cells were given F10 minus Thy containing 0.03 μCi/ml of [2-^{14}C]thymidine (56 mCi/mmol, Nuclear Dynamics, El Monte, Calif.). On the fourth day, after incubation in unlabeled medium for 1–2 hr, the cells were given F10 minus Thy containing 1.5×10^{-4} *M* unlabeled BrUdRib and 15 μCi/ml of [6-^{3}H]BrUdRib (27.6 Ci/mmol, New England Nuclear). Twenty hours later (day 5) the cells were incubated for 1–2 hr in nonradioactive F10 minus Thy containing 1.5×10^{-4} *M* BrUdRib which was then replaced. After a further doubling, the cells were harvested.

Cellular proteins were labeled with either 2 μCi/ml of [^{3}H]leucine (51 Ci/mmol, Amersham Searle) or 0.2 μCi/ml of [^{14}C]lysine (348 mCi/mmol, Amersham Searle) and 0.2 μCi/ml of [^{14}C]leucine (270 mCi/mmol, Amersham Searle) in medium containing one-half the normal concentration of unlabeled lysine and leucine.

Isolation of Chromatin. All manipulations were carried out on ice under a yellow (insect repellant) light to avoid wavelengths to which BrUdRib is sensitive and using plastic labware to minimize losses by adsorption.

Following a 30-sec rinse in cold 0.05% trypsin (Gibco) the bottles were incubated at 37°C in 20 ml of 0.08 *M* NaCl, 0.02 *M* EDTA (pH 8.0), 1 mg/ml of bovine serum albumin, and 0.05 *M* $NaHSO_3$ until detachment occurred. The pellet obtained by low-speed centrifugation was resuspended in 10 ml of 0.08 *M* NaCl–0.02 *M* EDTA (pH 8.0), mixed with 10 ml of 0.08 *M* NaCl, 0.02 *M* EDTA (pH 8.0), and 2% Triton X-100, and sheared for 30 sec (Sorvall Omnimixer 50-ml cup, 60 V, 30 sec). The nuclei were centrifuged through 1.7 *M* sucrose–0.08 *M* NaCl–0.02 *M* EDTA (pH 8.0) (197000*g*, 1 hr) and the pellets stored at −70°C. One bottle of cells yielded approximately 1.2×10^8 nuclei containing 2×10^5 cpm of [^{3}H]BrUdRib and 9×10^4 cpm of [^{14}C]thymidine.

To isolate chromatin, fresh or thawed nuclear pellets were slowly added to 100 volumes of distilled water and the mixture was stirred for 30 min at which time microscopic examination showed nuclear lysis to be complete. The chromatin was pelleted by centrifugation for 1 hr at 27000*g*, and sheared (Virtus 45 homogenizer, 5-ml cup, 30 V, 30 sec) in 1.2 ml of 10^{-4} *M* EDTA (pH 8.0). This material, at an optical density at 260 nm of 0.5, had an optical density at 450 mm of less than 0.04 and a protein to DNA ratio of 2.6 (Lowry et al., 1951; Burton, 1956).

Irradiation of Sheared Chromatin. Sheared chromatin was irradiated in a 1-cm quartz cuvette cooled by circulating ice-water. In some experiments, the cuvette was placed 10 cm from a Model 12 A90 Hanovia sun lamp and received a total incident dose of 3×10^4 ergs/mm^2 of unfiltered light in 4 min as measured by a Blak-Ray ultraviolet meter, Ultraviolet Products. In other experiments the sample was irradiated from both sides with 9×10^4 ergs/mm^2 of 313-nm light delivered in 6 hr by passing the light from two sun lamps through 313-nm band pass filters (Barr Associates, West Concord, Mass.). [Experiments to be described in a subsequent paper have used an improved 313-nm source; details may be obtained from the authors.] Following irradiation, 0.11 volume of SSC (0.15 *M* NaCl–0.015 *M* sodium citrate (pH 7.0)) was added and the chromatin incubated at 37°C for 10 min; this treatment appeared to improve the sucrose gradient separations.

Sucrose Gradients. The sample (0.2–0.4 ml) was layered on a 4.5-ml 5–20% linear sucrose gradient over a 0.2-ml 60% sucrose cushion and centrifuged in a SW50.1 rotor under conditions specified below. The sucrose was buffered with 0.1 × SSC. Fractions (0.25 ml) were counted in 10 ml of Bray's (1960) solution. Corrections for quenching and spillover of ^{14}C into the ^{3}H channel were applied using standard curves made with chromatin containing only a single label.

Preparative Cs_2SO_4 Density Gradient Centrifugation. The distribution of ^{3}H and ^{14}C labels in the HL and HH DNA was determined by preparative Cs_2SO_4 density gradient centrifugation as described by Flamm et al. (1966). The chromatin in 4% Sarkosyl NL-97 (Geigy) was brought to a refractive index of 1.3725 by the addition of saturated Cs_2SO_4 (in 0.1 × SSC) and centrifuged 20 hr at 19°C at 42000 rpm in a Beckman Type 65 rotor. Aliquots of each fraction on Whatman 3MM filter discs were washed with trichloroacetic acid and counted in a toluene-based scintillation fluid.

Analytical CsCl Density Gradient Centrifugation. The buoyant density of the HL and HH DNA was determined by CsCl density gradient centrifugation as described previously (Taichman and Rownd, 1975).

Dodecyl Sulfate Gel Electrophoresis of Chromatin-Associated Proteins. Sheared irradiated chromatin was incubated at 37°C for 2 hr with 0.054 volume of 1.25 *M* Tris-HCl (pH 6.8), 0.0108 volume of 1 *M* $CaCl_2$, and 0.0108 volume of staphylococcal nuclease (200 units/ml, Sigma); 100 μg of carrier chick histone was added and the protein precipitated by the addition of 0.27 volume of 100% trichloroacetic acid. The precipitate was washed once in acidified acetone (0.1 ml of concentrated HCl in 100 ml of acetone) and air dried. The precipitate was dissolved in sample buffer and electrophoresed as described by Laemmli (1970) in 15% (w/v) acrylamide gels using 1.6% (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 Triton-xylene scintillation fluid (Anderson and McClure, 1973).

Results

BrUdRib-Substituted DNA. To prepare chromatin containing ^{14}C-labeled HL DNA and ^{3}H-labeled HH DNA, CHO cells were cultured as described in Figure 1. Prepara-

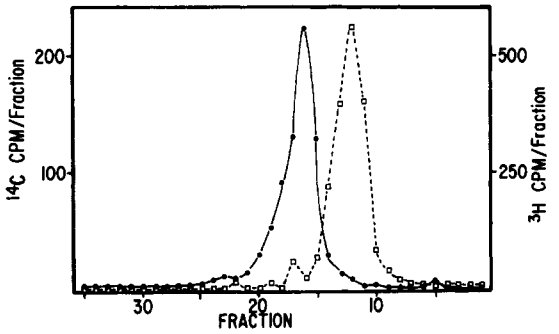

FIGURE 2: Preparative Cs_2SO_4 density gradient centrifugation. The DNA obtained from cells labeled with [^{14}C]thymidine and [^{3}H]BrUdRib according to Figure 1 was centrifuged to equilibrium in Cs_2SO_4 density gradients as described in Methods. Density increases from left to right. (□) ^{3}H cpm; (●) ^{14}C cpm.

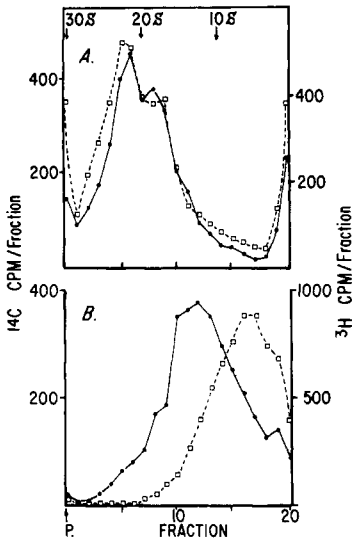

FIGURE 3: Sedimentation of HL and HH DNA. (A) DNA from unirradiated chromatin. (B) DNA from chromatin that had been irradiated with 3×10^4 ergs/mm^2 of unfiltered ultraviolet light. In both cases chromatin containing ^{14}C-labeled HL DNA and ^{3}H-labeled HH DNA was treated with Pronase and dodecyl sulfate as described in the text. The digest was then centrifuged in 5-20% sucrose gradients at 20°C at 45000 rpm for 3 hr in an SW 50.1 rotor with λ DNA as a size marker (not shown). "P" on the abcissa indicates the pellet. (□) ^{3}H cpm; (●) ^{14}C cpm.

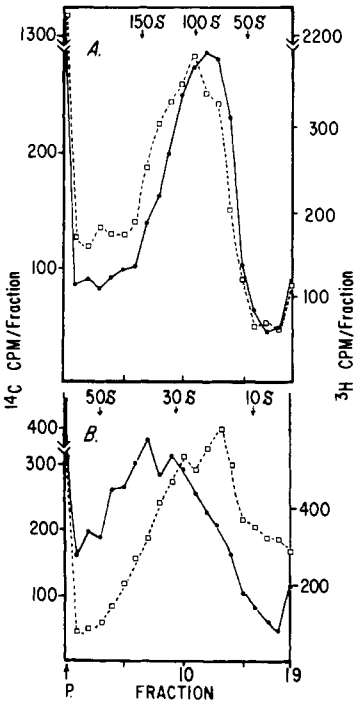

FIGURE 4: Sedimentation of chromatin containing HL and HH DNA. (A) Unirradiated chromatin was centrifuged for 1 hr. (B) Chromatin irradiated with 3×10^4 ergs/mm^2 was centrifuged for 4 hr. [The longer time was necessary to fully display the irradiated chromatin on the gradient.] Chromatin containing ^{14}C-labeled HL DNA and ^{3}H-labeled HH DNA was centrifuged in 5-20% sucrose gradients at 37000 rpm at 4°C in an SW 50.1 rotor. Note that the scale of s values is different in the two parts of the figure. (□) ^{3}H cpm; (●) ^{14}C cpm.

tive Cs_2SO_4 density gradient centrifugation of the DNA moiety of the chromatin confirmed the presence of a denser ^{3}H band and a lighter ^{14}C band (Figure 2). Analytical centrifugation of the same DNA in CsCl indicated the densities of the two bands to be 1.748 and 1.797 g/ml, respectively, thus confirming the predicted distribution of the label. Using these densities the mole fraction of BrUdRib in each substituted strand can be calculated to be 0.28 (Rownd, 1967). The density of unsubstituted LL DNA was found to be 1.702 g/ml which corresponds to a mole fraction of thymidine of 0.28 (using the formulas developed by Schildkraut et al., 1962). The value of 0.28 obtained in both cases indicates that virtually all of the thymidine has been replaced by BrUdRib in the H strands of the HL and HH DNA.

Separation of the HL and HH DNA on Sucrose Gradients. To determine whether differential breakage of HL and HH DNA occurs when chromatin is irradiated with ultraviolet light, 0.3 ml of sheared chromatin was irradiated with 3×10^4 ergs/mm^2 of unfiltered ultraviolet light. A second sample, kept in the dark, served as the unirradiated control. The DNA was centrifuged through a 5-20% linear sucrose gradient after removal of the chromatin protein by Pronase (0.5 mg/ml) and sodium dodecyl sulfate (5%). Control experiments established the efficacy of this treatment in removing the proteins from the DNA. Figure 3A demonstrates that after these procedures, HL and HH DNA from the unirradiated chromatin both sediment as single broad bands (Figure 3A) with sedimentation coefficients ranging from 15 S to 25 S (mean 20 S). The molecular weights of these DNA molecules range from 3 to 15×10^6 (mean 7×10^6) as calculated using the equation of Studier (1965) and λ phage DNA as a marker. The unirradiated HH DNA sediments slightly faster than the unirradiated HL DNA; this is a consistent finding in unirradiated samples and is presumably caused by the greater density of HH DNA. Following irradiation with unfiltered ultraviolet light, there is a significant separation of the HL and HH DNA on the sucrose gradient (Figure 3B). The molecular weight of the HH DNA has decreased markedly to a mean value of 0.3×10^6 while that of HL DNA has fallen to a mean value of 1.4×10^6. Thus, irradiation of chromatin with unfiltered ultraviolet light leads to the differential fragmentation of HL and HH DNA as predicted.

Separation of Chromatin Containing HL and HH DNA. Figure 4 compares the sedimentation behavior of standard sheared chromatin before and after irradiation with 3×10^4 ergs/mm^2 of unfiltered ultraviolet light. Unirradiated chromatin sediments as a broad band with sedimentation coefficients of 50 S to 150 S (mean 100 S) (Figure 4A). After irradiation, the s values of chromatin containing HL DNA

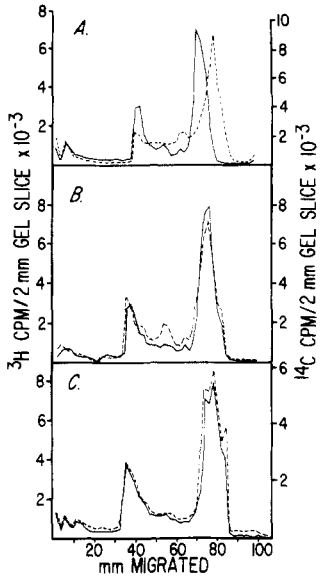

FIGURE 5: Effect of irradiation on the electrophoretic behavior of chromatin-associated proteins. (A) BrUdRib-substituted chromatin containing proteins labeled with [^{3}H]lysine and [^{3}H]leucine was irradiated with 3×10^4 ergs/mm^2 of unfiltered ultraviolet light. The [^{3}H]chromatin was then mixed with unirradiated BrUdRib substituted chromatin containing proteins labeled with [^{14}C]lysine and [^{14}C]leucine. The proteins were then isolated, electrophoresed in 15% dodecyl sulfate gels, and analyzed as described in Methods. (B) The same as A except that the [^{3}H]chromatin was irradiated with 9×10^4 ergs/mm^2 of 313-nm light. (C) The same as A except that neither chromatin was irradiated. The direction of migration is from left to right. Histones (except I) migrate in the region of fractions 65–80. (- - -) ^{3}H cpm; (—) ^{14}C cpm.

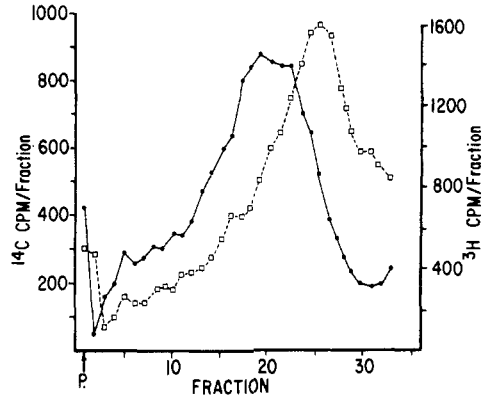

FIGURE 6: Sedimentation of chromatin irradiated with 313-nm light. Chromatin containing ^{14}C-labeled HL DNA and ^{3}H-labeled HH DNA was irradiated with 9×10^4 ergs/mm^2 of 313-nm light. 0.6 ml of sample was layered on a 30-ml 9–30% (w/v) linear sucrose gradient (in 0.1 × SSC) over a 1.5-ml cushion of 60% sucrose (in 0.1 × SSC). The material was centrifuged for 16 hr at 17000 rpm at 4°C in an SW 27 rotor. 1.3-ml fractions were collected in scintillation vials and counted as described in Methods. (□) ^{3}H cpm; (●) ^{14}C cpm.

range from 15 S to 50 S (mean 35 S) while those of chromatin containing HH DNA fall between 10 S and 40 S (mean 25 S). This experiment establishes that after ultraviolet irradiation the two types of chromatin can be partially separated on neutral sucrose gradients. This result has been achieved many times using gradients in both the SW 50.1 and SW 27 rotors.

Effect of Ultraviolet Light on the Electrophoretic Behavior of Chromatin-Associated Proteins. The electrophoretic behavior of proteins isolated from irradiated chromatin was compared with that of proteins from unirradiated material on dodecyl sulfate polyacrylamide gels (Figure 5). ^{3}H-labeled proteins from chromatin irradiated with unfiltered ultraviolet light were found to migrate further than the unirradiated control, suggesting that the proteins are partially fragmented by the light. To avoid this alteration we irradiated chromatin with 313-nm light. This wavelength has been shown to optimize the ratio of damage to BrUdRib DNA to damage to unsubstituted DNA (Boyce and Setlow, 1963; Setlow and Boyce, 1962).

Figure 5B shows that proteins from chromatin irradiated with 9×10^4 ergs/mm^2 of 313-nm light migrate with the same mobilities as those from an unirradiated control. There is a slight change in the relative proportions of the proteins of different molecular weight which could be caused by differential recoveries resulting from some cross-linking of protein to DNA. However, 313-nm light clearly causes less damage to the chromatin-associated proteins than unfiltered light.

In Figure 6 chromatin containing HL and HH DNA is shown to sediment as two distinct bands after irradiation with 9×10^4 erg/mm^2 of 313-nm light. This establishes that the 313-nm light which causes minimal protein alterations can produce differential fragmentation of the chromatin.

Discussion

The experiments described establish that irradiation of chromatin containing BrUdRib-substituted DNA with 313-nm ultraviolet light leads to fragmentation of the chromatin and that chromatin containing HH DNA breaks into smaller pieces than chromatin containing HL DNA. This differential fragmentation permits the separation of the two types of chromatin on neutral sucrose gradients. The chromatin-associated proteins appear to be damaged only slightly by the 313-nm light.

Hancock (1970) achieved a partial separation of chromatin containing normal and iodouracil-substituted DNA by isopycnic centrifugation in CsCl. To prevent the dissocation of proteins in the high salt, the chromatin was cross-linked with formaldehyde making further analysis difficult. The technique reported here uses an analogue of thymidine less toxic than iodouracil and achieves a better separation of chromatin under conditions which do not require cross-linking.

The procedure was designed for use in studying the distribution of proteins during chromatin replication; these experiments are now in progress. This procedure should also be useful for studying the exchange of chromatin-associated proteins and for isolating newly replicated chromatin.

Double-stranded breaks in DNA occur in human kidney cells that have incorporated BrUdRib and then been irradiated with ultraviolet light (Smets and Cornelius, 1971), but there has been no published report that chromatin is fragmented by the same treatment. Our results show that double-stranded breaks in the DNA lead to fragmentation of interphase chromatin molecules. This phenomenon is likely to be the cause of the chromatid breaks observed in cells that have incorporated BrUdRib and have been irradiated with fluorescent light (Puck and Kao, 1967). Ultraviolet light was also shown in this study to alter the electrophoretic behavior of chromatin proteins. This observation suggests that such light, in addition to its effect on DNA, may possibly alter gene expression by its direct effect on the proteins in chromatin.

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