The use of ultraviolet light in the fractionation of chromatin containing unsubstituted and bromodeoxyuridine-substituted DNA

Lorne B. Taichman

Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook, Stony Brook, NY 11794, USA

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ABSTRACT

Two procedures are described for the fractionation of chromatin containing unsubstituted (LL) DNA and DNA unifilarly substituted with bromodeoxyuridine (HL). The two procedures rely upon the sensitivity of bromodeoxyuridine-containing DNA to UV light to induce either strand breakage or protein crosslinking. When a mixture of LL and HL chromatin is irradiated with UV light, the HL DNA fragments into molecules of smaller molecular weight than the LL DNA and crosslinks more chromosomal protein than the LL DNA. LL and HL chromatin can be fractionated on the basis of size by centrifuging through a neutral sucrose gradient. The HL DNA-protein adducts that are generated by the UV light have a unique buoyant density and may be isolated by isopycnic centrifugation in Cs$_2$SO$_4$. The ability to fractionate LL and HL chromatin permits certain studies on the structure of replicating chromatin.

INTRODUCTION

It is generally believed that the composition and arrangement of chromosomal proteins determines the genetic activity of the associated segments of DNA. The exploration of this relationship rests upon techniques for the fractionation of chromatin. Some success has been achieved in the separation of active and inactive chromatin$^1$, euchromatin and heterochromatin$^2$, and normal and density labeled chromatin$^3$. The separation of dense chromatin from normal chromatin has been useful in defining the gross structure of nascent chromatin$^3,4,5$ and elucidating the distribution of chromosomal proteins over multiple cycles of cell replication$^6,7,8$. The density of chromatin can be increased by the incorporation of bromodeoxyuridine (BrdUrd) or iododeoxyuridine into DNA and $^{15}$N, $^{13}$C and $^2$H labeled amino acids into chromosomal proteins$^3$. The dense chromatin can be partially separated from normal chromatin in CsCl density gradients if the chromatin is fixed with formaldehyde to prevent dissociation of the proteins. The usefulness of the technique is limited because formaldehyde can
induce histone redistribution in the chromatin and the separation achieved is minimal. To increase the density difference between substituted and unsubstituted chromatin, portions of the chromosomal proteins have been removed either by limited proteolysis or by salt elution of H1 histone and nonhistone proteins. These modifications impose additional limitations on the technique.

Tsichman and Freedlender have developed a different approach to the fractionation of BrdUrd-containing chromatin that utilizes the sensitivity of BrdUrd to ultraviolet (UV) light. When chromatin containing a mixture of unifilarly (HL) and bifilarly (HH) substituted DNA is irradiated with UV light, the HH chromatin fragments into smaller pieces than the HL chromatin. The HL and HH chromatin molecules can then be partially resolved on the basis of size by centrifugation in neutral sucrose gradients. UV light causes single stranded nicks mainly in the substituted strand of DNA, and double stranded breaks are produced in the HH molecule at sites where single stranded nicks occur close together in complementary strands. The separation of HL and HH chromatin has limited uses in that cells must be exposed to BrdUrd for more than one cycle of replication to generate HH DNA. Two new techniques are reported here for the fractionation of chromatin containing unsubstituted (LL) DNA and HL DNA. Both techniques utilize the sensitivity of BrdUrd to UV light, one is a modification of the procedure previously described and the second is a new procedure that involves crosslinking of proteins selectively to the HL DNA and the isolation of HL DNA-protein adducts in Cs2SO4 density gradients.

MATERIALS AND METHODS

CHO cells were grown in 100 mm plastic culture dishes under conditions previously described. The doubling time was 24 hours in normal medium and 36 hours in BrdUrd-containing medium. To label DNA, cells were grown in F10 medium lacking thymidine (dThd) and supplemented with 0.05μCi/ml thymidine-1-C (51.3mCi/mM, New England Nuclear) or with a mixture of 15μCi/ml 5-bromodeoxyuridine (6-3H) (22.8 Ci/mM, New England Nuclear) and 1.5 x 10^-4M unlabeled BrdUrd. To label proteins, the concentration of lysine and leucine in F10 was reduced to one third normal and the medium supplemented with 1.0μCi/ml of L-[5,5(n)-3H]lysine (21 Ci/mM, Amersham/Searle) and 1.0μCi/ml L-[4,5-3H] leucine (50 Ci/mM, Amersham/Searle) or 0.2μCi/ml L-lysine(14C(U)) (306mCi/mM, New England Nuclear) and 0.2μCi/ml
L-leucine \( [^{14}\text{C}(\text{U})] \) (325mCi/mM, New England Nuclear).

Chromatin was isolated as described previously\(^{10}\) with two changes: first, phenylmethylsulfonylfluoride (10\(^{-4}\)M) was added to all solutions and sodium bisulfite omitted; and second, the monolayers of cells were dislodged from the surface by incubating the cells for 10 minutes at 37\(^{\circ}\)C in saline-EDTA buffer with no prior exposure to trypsin. Isolated chromatin in 30mm plastic dishes were exposed to the UV light from a 275 watt mercury lamp (Hanovia, Model 30410) at a flux of 1.6 \( \times \) 10\(^{2}\) ergs/sec/mm\(^{2}\) as measured with a short-wave Blak Ray ultraviolet Meter (Model J-225, Ultraviolet Products). To prevent heating, the dish was floated in icewater. Irradiated chromatin was centrifuged in 5-20\% neutral sucrose gradients as described\(^ {10}\). To isolate HL DNA-protein adducts, irradiated chromatin was treated with 1\% Sarkosyl (Giegy) at 37\(^{\circ}\)C for 30 minutes, brought to a volume of 6\text{ml} and a refractive index of 1.3608 by the addition of SSC/10 and SSC/10 saturated with \( \text{Ca}_2\text{SO}_4 \) and centrifuged at 44,000 rpm for 36 to 40 hours at 20\(^{\circ}\)C in a 50Ti rotor (Beckman Instruments). Gradient fractions were counted in a toluene based cocktail\(^ {10}\).

RESULTS

Fractionation of LL and HL chromatin in sucrose gradients. When the technique for fractionation of HL and HH chromatin was developed\(^ {10}\), it was anticipated that HL DNA would not fragment appreciably when exposed to UV light. Since the L strand is 100-1000 fold less sensitive to photoinduced strand breakage than the H strand\(^ {11}\), it was thought that the molecular weight of irradiated HL DNA would be one to two orders of magnitude greater than the HH DNA. However, the size difference was only about 4 fold. The reasons for this are unclear. However, the fact that HL chromatin does fragment at an appreciable rate suggests that LL and HL chromatin can be fractionated in the same way as HL and HH chromatin. To test this, two cultures of CHO cells were labeled so that one contained a mixture of \( ^{14}\text{C}-\text{HL DNA} \) and \( ^{3}\text{H}-\text{HL DNA} \) and the other culture contained a mixture of \( ^{14}\text{C}-\text{HL DNA} \) and \( ^{3}\text{H}-\text{HH DNA} \). The labeling protocols are illustrated in Figure 1 and the verification of the effectiveness of this labeling regime has been documented\(^ {10}\). The chromatin was isolated from each culture, irradiated with 10\(^{5}\) ergs/mm\(^{2}\) of UV light and sedimented in a linear 5-20\% sucrose density gradient (Figure 2). HH, HL and LL chromatin sediment as broad but distinct bands with mean sedimentation coefficients \((S_{20},w)\) of 2031.
Figure 1. Labeling protocol for CHO cells. To generate mixtures of \(^{14}\)C-LL and \(^{3}\)H-HL DNA in one culture and \(^{14}\)C-HL and \(^{3}\)H-HH DNA in a second culture, CHO cells were labeled with \(^{14}\)C-dThd and \(^{3}\)H-BrdUrd as shown. The DNA is represented by two vertical lines; a solid line indicates an unsubstituted strand and a dashed line indicates a BrdUrd-substituted strand.

22, 50 and 70 respectively. The sedimentation coefficients were calculated using formulae developed by McEwen\(^ {12}\). The results clearly show that LL and HL chromatin can be resolved in sucrose gradients and that the degree of separation achieved for LL and HL chromatin is the same as that achieved for HL and HH chromatin. As a preparative procedure, one could recover in

Figure 2. Sedimentation of UV-irradiated chromatin in sucrose gradients. Chromatin was isolated from cells labeled according to the protocol of Figure 1., irradiated with \(10^3\) ergs/mm\(^2\) of UV light and sedimented at 4°C for 75 min. in a linear 5-20% gradient at 48,000 rpm in an SW50.1 rotor. The upper panel shows \(^{14}\)C-HL and \(^{3}\)H-HH chromatin and the lower panel shows \(^{14}\)C-LL and \(^{3}\)H-HL chromatin. Fraction 1 is the pellet and sedimentation is from right to left.
fractions 12 to 16 of the gradient of Figure 2B, 40% of the total HL chromatin contaminated by 12% of LL chromatin. There has been roughly a threefold enrichment of HL chromatin.

Isolation of HL DNA-protein adducts. The isolation of HL DNA-protein adducts from cells containing both LL and HL DNA rests upon three facts: first, when cells are exposed to UV light stable DNA-protein adducts are formed; second, this reaction is enhanced if BrdUrd is incorporated into the DNA; and third, a covalently bonded adduct of DNA and protein has a buoyant density intermediate between free DNA and free protein depending on the relative amounts of each. It seems reasonable to predict that if HL DNA crosslinks more protein than the LL DNA, the HL DNA-protein adducts thus formed would equilibrate at a unique position in density gradients. To test this hypothesis, cells containing both \(^{14}\)C-LL and \(^{3}\)H-HL chromatin were generated by labeling a subconfluent culture for one hour with \(^{14}\)C-dThd, followed by a one-hour period in unlabeled medium and finally for 30 minutes with \(^{3}\)H-BrdUrd. Since the cells are not synchronized, some will contain only \(^{14}\)C-LL chromatin and others only \(^{3}\)H-HL chromatin. However, most will contain segments of \(^{14}\)C-LL chromatin and \(^{3}\)H-HL chromatin. Chromatin was isolated and the size of the associated DNA was measured by treating the chromatin for 3 hr at 37°C with proteinase K and sedimenting in a neutral sucrose gradient at 20°C. Using T7 DNA as a size marker, the weight average molecular weight of the DNA was found to be 2.5 x 10^7. Based on an average rate of DNA synthesis of 1500 base pairs/min, the labeled segments of DNA would range in molecular weight from 3-6 x 10^6. To fractionate the DNA-protein adducts, the isolated chromatin was treated with Sarkosyl and centrifuged to equilibrium in Cs_2SO_4 as described in Methods. Unirradiated \(^{14}\)C-LL and \(^{3}\)H-HL DNA equilibrate in Cs_2SO_4 at buoyant densities of 1.422 and 1.455 g/cm^3, respectively (Figure 3A). However, when the chromatin mixture is irradiated with 5.6 x 10^4 ergs/mm^2 of UV light, the relative positions of the LL and HL DNA bands are reversed (Figure 3B). The \(^3\)H-HL DNA forms a broad band with a buoyant density at its peak of 1.275 g/cm^3 and the \(^{14}\)C-LL DNA bands at a higher density of 1.370 g/cm^3. To show that this change in buoyant density results from the crosslinking of protein, a portion of the irradiated sample was treated with 100 µg/ml of proteinase K for 3 hours at 37°C and centrifuged Cs_2SO_4. The LL and HL DNA formed two distinct bands that were slightly broader but with the same buoyant densities as LL DNA and HL DNA from unirradiated cells (Figure 3C). We conclude that HL DNA crosslinks more
protein than LL DNA and that the HL DNA-protein adducts may be recovered free of LL DNA and noncrosslinked protein in a density gradient of Cs₂SO₄.

To determine what fraction of chromosomal proteins originally in HL chromatin are recovered in HL DNA-protein adducts, the following experiment was performed: One culture of CHO cells was grown for 36 hours in medium containing unlabeled dThd, ¹⁴C-lysine and ¹⁴C-leucine. A second culture was labeled for 36 hours with unlabeled BrdUrd (1.5 x 10⁻⁴M), ³H-lysine
and $^3$H-leucine. The first culture contained LL chromatin with $^{14}$C-protein and second contained HL chromatin with $^3$H-protein. The two cultures were combined, their chromatin isolated and centrifuged in Cs$_2$SO$_4$. In the unirradiated mixture virtually all the $^{14}$C-protein and $^3$H-protein float at the meniscus (Figure 4A). In Figure 4B the chromatin mixture has been irradiated ($5.6 \times 10^4$ ergs/mm$^2$) and a significant fraction of the $^3$H-proteins and some of the $^{14}$C-proteins band where the DNA-protein adducts are usually found. To determine the precise location of HL DNA-protein complexes, two additional cultures were labeled in parallel with the same medium except that one received $^{14}$C-dThd and the other $^3$H-BrdUrd instead of the labeled amino acids. This labeling scheme generated cells with $^{14}$C-LL

Figure 4. Chromatin in Cs$_2$SO$_4$. A, LL chromatin containing $^{14}$C-protein was mixed with HL chromatin containing $^3$H-protein, treated with Sarkosyl and centrifuged to equilibrium in Cs$_2$SO$_4$. B, as in A. The chromatin mixture was irradiated prior to the Sarkosyl treatment. C, $^{14}$C-LL chromatin (DNA labeled) was mixed with $^3$H-HL chromatin (DNA labeled), irradiated and treated as in A.
DNA and $^3$H-HL DNA, respectively. The chromatin from this mixture was irradiated and centrifuged in parallel with the protein labeled material (Figure 4C). The HL DNA-protein complexes are located primarily in fractions 9 to 16 and this adduct ("A") region has been demarcated in Figure 4B by the arrow. The A region contains 81% of the $^3$H-HL DNA, 50% of the $^3$H-chromosomal proteins, 17% of the $^{14}$C-LL DNA and 20% of the $^{14}$C-chromosomal proteins. The HL DNA-protein complexes in the A region are contaminated by a small amount of LL DNA-protein complexes. The technique of UV irradiation and Cs$_2$SO$_4$ density centrifugation is useful in the partial purification of proteins associated with segments of chromatin containing HL DNA.

DISCUSSION

Techniques are reported here for the fractionation of LL and HL chromatin in neutral sucrose gradients and for the isolation of HL DNA-protein adducts in Cs$_2$SO$_4$ density gradients. Both techniques utilize the sensitivity of BrdUrd-containing DNA to UV light, in the former instance to induce breaks in the HL molecule of chromatin and in the latter instance to induce covalent crosslinking of HL DNA to the associated protein. It should be pointed out that both reactions occur in irradiated chromatin and that each technique starts with the same material and exploits only one of these reactions. These experiments have utilized mixtures of $^{14}$C-LL and $^3$H-HL chromatin and from the design of the experiments the labeled LL and HL molecules would be separate from one another, not on adjacent segments. The purification of HL chromatin is not complete with either technique. Using differential fragmentation and sedimentation through sucrose, a threefold purification HL chromatin can be achieved. Using the crosslinking reaction and the centrifugation in Cs$_2$SO$_4$, the chromosomal proteins associated with HL DNA can be purified about two and one-half fold.

The techniques described in this publication make possible certain studies on the structure of replicating chromatin. For example, the chromosomal proteins found on newly synthesized DNA could be defined by labeling cells briefly with BrdUrd, irradiating the chromatin and fractionating the HL DNA-protein adducts in Cs$_2$SO$_4$. Another example would be to determine if different molecules of DNA "share" chromosomal protein(s). If LL DNA and HL DNA are in contact with the same chromosomal protein, then UV light may induce the formation of a complex consisting of LL DNA, HL DNA and protein. Current work in this laboratory is exploring these uses.
Previous work\textsuperscript{7} has demonstrated that the proteins in irradiated chromatin can be analyzed electrophoretically if monochromatic 313nm light is used instead of broad spectrum UV light and if the associated DNA is hydrolyzed with DNase I. All five histones and nonhistone proteins are known to cross-link to unsubstituted DNA when nucleosomes of various size classes are exposed to UV light\textsuperscript{19,20}. Histones H1, H2A and H2B crosslink more readily than the other histones\textsuperscript{21}.

There is one advantage in using UV light to crosslink proteins that should be mentioned. Since the crosslinking reaction can be done by irradiating intact cells, problems associated with movement or rearrangement of proteins during chromatin isolation\textsuperscript{22,23,24,25} can be avoided. The specificity of this photochemical reaction in nucleoprotein complexes has been documented\textsuperscript{26,27,28}.

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**REFERENCES**