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# Affinity Isolation of Active Murine Erythroleukemia Cell Chromatin: Uniform Distribution of Ubiquitinated Histone H2A Between Active and Inactive Fractions

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**Abstract** This laboratory recently reported the development of a biotin-cellulose/streptavidin affinity chromatography method based on the DNase I sensitivity of active chromatin to isolate a DNA fraction from murine erythroleukemia (MEL) cells that is more than 15-fold enriched in active genes (Dawson et al.: *Journal of Biological Chemistry* 264:12830–12837, 1989). We now report the extension of this technique to isolate and characterize chromatin that is enriched in active genes. In this approach, DNA in nuclei isolated from MEL cells was nicked with DNase I at a concentration that does not digest the active beta-globin gene, followed by repair of the nicks with a cleavable biotinylated nucleotide analog, 5-[(N-biotin-amido)hexanoamido-ethyl-1,3'-dithiopropionyl-3-aminoallyl]-2'-deoxyuridine 5'-triphosphate (Bio-19-SS-dUTP), during a nick-translation reaction. After shearing and sonication of the nuclei to solubilize chromatin, chromatin fragments containing biotin were separated from non-biotinylated fragments by sequential binding to streptavidin and biotin cellulose. The bound complex contained approximately 10% of the bulk DNA. Reduction of the disulfide bond in the biotinylated nucleotide eluted approximately one-half of the affinity isolated chromatin. Hybridization analysis of DNA revealed that whereas inactive albumin sequences were equally distributed among the chromatin fractions, virtually all of the active beta-globin sequences were associated with chromatin fragments which had bound to the affinity complex. Western blot assessment for ubiquitinated histones revealed that ubiquitinated histone H2A (uH2A) was uniformly distributed among active (bound) and inactive (unbound) chromatin fractions.

**Key words:** chromatin structure, gene expression, streptavidin-biotin affinity chromatography

The fundamental unit of chromatin structure—the nucleosome—is composed of approximately 200 bp of DNA wrapped around a histone octamer containing two molecules each of histones H2A, H2B, H3, and H4. Although the historical concept of nearly absolute evolutionary conservation of the histones implied that nucleosomes were also homogeneous in composition, the now well-established findings that histones contain variations in primary structure and may be extensively modified indicate that nucleosomes can be very heterogeneous [1]. The functional advantage(s) of altered nucleosomes remains controversial, mostly due to the unavailability of methods to accurately assess the effects of altered structural chromatin proteins on genome function. Thus, only indirect evidence for the role(s) of these proteins can be obtained, for example, by assessing whether nucleosomes containing specifically altered histones are enriched in transcriptionally active or inactive chromatin.

An unequivocal assignment of specifically altered histones to functional domains of the genome requires a reliable method to precisely separate active and inactive chromatin fractions. To this end, fractionation methods have been employed that usually involve the manipulation of isolated nuclei with endogenous [2] or exogenous enzymes. The latter approach includes limited digestion with DNase II [3] or, most frequently, micrococcal nuclease [4–8]. Unfortunately, these methods yield fractions of variable enrichment, which is apparently dependent upon cell type and salt concentration [9,10]. Digestion with restriction endonucleases has also been unsatisfactory because these enzymes ap-

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pear to preferentially solubilize chromatin containing repetitive DNA [11,12].

Recently, an affinity method for isolating transcribing chromatin from a micrococcal nuclease-generated fraction of rat liver nuclei was reported in which relaxed, active nucleosomes containing exposed histone H3 sulfhydryl groups were bound to an organomercurial-agarose support [13]. In the current study we have isolated active chromatin from differentiating murine erythroleukemia (MEL) cells using an affinity method that exploits the sensitivity of active genes to DNase I [14,15]. In this approach, nucleosomes in active globin gene domains were nicked with DNase I, repaired with a biotinylated nucleotide analog (Biotin-19-SS-dUTP), and isolated using streptavidin/biotin cellulose affinity chromatography. We have previously described the use of this technique to fractionate purified DNA [16].

Among the post-translational modifications to histones, perhaps the most extensive is the ligation of the peptide ubiquitin to histone H2A and, to a lesser extent, H2B. Although ubiquitination of cytoplasmic proteins has been shown to mark them for degradation [17], the function of histone ubiquitination remains controversial [18]. Whereas reports have indicated that ubiquitinated histones reside in transcriptionally active nucleosomes [6,19], it has also been reported that the degree of H2A ubiquitination in HeLa cells does not correlate with the rate of transcription [20]. Moreover, ubiquitinated histones do not co-enrich with active DNA sequences in micrococcal nuclease-generated fractions of plasmacytoma cell [21] or, as previously reported from this laboratory, myotube chromatin [10]. In the studies described here, we have assessed oligonucleosomes in affinity chromatographed fractions of active chromatin from MEL cells for enrichment of ubiquitinated histones. In accord with the latter findings, these determinations indicate that ubiquitinated histones are randomly distributed among active and inactive chromatin.

## EXPERIMENTAL PROCEDURES

### Cell Culture

MEL cells of clone 745 from the Human Genetic Mutant Cell Repository (Camden, NJ) were maintained in Eagle's minimum essential medium supplemented with 50 μg/ml gentamicin, 10% fetal bovine serum, and 5% defined calf serum. To induce differentiation, log-phase cells were treated with 5 mM hexamethylene bisacetamide (HMBA) and grown for 5–6 days. Staining for hemoglobin [22] revealed 90–95% benzidine positive cells. To prelabel total nuclear DNA, $^{3}$H-thymidine (32 Ci/mmol; ICN) or $^{14}$C-thymidine (58 mCi/mmol; ICN) was added for the last 60 h of cell growth (about two generations) at 0.025 or 0.01 μCi/ml, respectively. To label histones, cells were grown in the presence of 2.5 μCi/ml $^{3}$H-lysine (77 Ci/mmol; Amersham) and 2.5 μCi/ml $^{3}$H-arginine (50 Ci/mmol; Amersham) for the last 60 h. Prelabeling with these radioisotopes, alone or in combination, had no effect on cell viability, growth rate, or DNA integrity.

### Nicking and Repair of Isolated Nuclei

MEL cell nuclei were isolated exactly as described previously [16] except that 0.25 mM iodoacetamide (Aldrich, Milwaukee, WI) was added to the nuclear lysis buffer to inhibit ubiquitin isopeptidases. Washed nuclei were resuspended in nick translation buffer [NT buffer; 16] to a final concentration of 77 × $10^{6}$/ml and nicked with 3.0 units/ml DNase I (2,200 units/mg; Pharmacia LKB Biotechnology, Inc.) for 10 min at 25°C. Nicks were repaired in NT buffer containing 20 μM dATP, dGTP, Bio-19-SS-dUTP, 10 μM dCTP, and 50 μCi/ml alpha-$^{32}$P-dCTP (3300 Ci/mmol; ICN); 100 units/ml *Escherichia coli* DNA polymerase I (Molecular Biology Resources, Milwaukee, WI) were added to start the reaction, which continued for 60 min at 15°C as previously described [16].

### Solubilization of Chromatin

Nicked and repaired nuclei were sedimented (2,000*g*, 2 min), washed twice with NT buffer, and resuspended at a concentration of 70 × $10^{6}$/ml in TE (10 mM Tris, pH 7.0; 1 mM EDTA). Nuclei were swollen for 20 min at 0°C and pelleted at 5,000*g* for 5 min. After discarding the supernatant, the pellet was resuspended in distilled water (DW) for 45 min, sheared with a Virtis 23 homogenizer at one-half maximum output for 1 min, followed by sonication for 15 sec at a setting of "4" using a model 185 Branson Sonifier Cell Disruptor equipped with a microtip. The solubilized, fragmented chromatin was adjusted to 10 mM Tris (pH 7.0), 1 mM EDTA. To estimate the dissociation of protein from DNA during chromatin solubilization, sheared and sonicated chromatin fragments that had been prelabeled with $^{14}$C-thymidine and $^{3}$H-

lysine/arginine were separated on sucrose gradients, and the co-migration of DNA and protein was determined. Fragmented chromatin (0.5 ml) in TE (pH 7.0) was layered over an 11 ml linear 5–25% sucrose gradient with a 50% sucrose cushion in TE (pH 7.0) and centrifuged 16 h at 30,000 rpm (100,000*g*) in an SW41 Ti rotor at 4°C. Fractions (0.5 ml) were collected and counted.

### Chromatin Affinity Chromatography

Biotin cellulose was prepared and assayed as previously described [16]. Picomoles of biotin incorporated into chromatin were estimated from the picomoles of $^{32}$P-dCTP incorporated during the repair reaction. Fragmented, biotinylated chromatin was incubated in TE (pH 7.5) with a 25-fold picomolar excess of streptavidin (SA) while rotating for 30 min. A 1,000-fold picomolar excess of biotin cellulose (relative to picomoles of biotinylated DNA) was mixed with the SA-chromatin complex for an additional 30 min. After sedimenting the mixture for 2 min at 1,000*g*, the unbound chromatin in the supernatant was removed and saved. The pellet was resuspended in TE (pH 7.5), centrifuged as above, and the supernatants were pooled as the unbound fraction (U). The biotinylated chromatin that bound to the pelleted complex was isolated by resuspending in TE (pH 8.3) and adding 50 mM dithiothreitol (DTT; BRL) to reduce the disulfide bond in Bio-19-SS-dUTP. After 15 min, the suspension was pelleted at 1,000*g* for 2 min and the supernatant containing the biotinylated chromatin was removed. The pellet was reincubated with DTT and pelleted and the supernatants were pooled as the bound and released fraction (B). Chromatin that was not released with DTT was termed the bound and retained fraction (R). Protein associated with retained (R) chromatin was obtained by extracting for 30 min with 0.25 N HCl at 4°C.

### DNA Purification, Electrophoresis, and Hybridization

Pooled fractions of unbound and bound chromatin were concentrated in a Speed-Vac (Savant), adjusted to 0.1% SDS, 100 mM NaCl and digested with 200 μg/ml proteinase K (Boehringer Mannheim) overnight at 37°C. Chromatin retained by the resin was obtained by resuspending in proteinase K buffer (5 mM Tris, pH 7.6, 100 mM NaCl, 7.5 mM EDTA, 5 mM EGTA, 0.2% SDS) and digesting with proteinase K. The DNA was purified by organic extraction and digestion with RNase A as previously described [16]. Total nuclear DNA (T), as well as DNA in bound and released (B), unbound (U), and bound and retained (R, resin) fractions were electrophoresed on 1% agarose gels and transferred to Zeta Probe nylon membrane (BioRad) under alkaline conditions as previously described; in addition, DNA samples were directly dot-blotted onto Zeta Probe [16].

Transfers were prehybridized for 48 h at 70°C in blocking buffer containing 2% bovine serum albumin (Sigma), 0.5 M $Na_2HPO_4$, (pH 7.2), 1.0 mM EDTA, 5% SDS, and 2% dextran sulfate. Probes were nick-translated as previously described [16] with alpha-$^{32}$P-dCTP; hybridization was carried out in the same buffer for 20 h at 70°C. Hybridized blots were washed and autoradiographed as described previously [16].

Probes for transcriptionally inactive genes, pAFP 2 (alpha-fetoprotein cDNA) and pmalb 2 (albumin cDNA), were provided by Dr. S.M. Tilghman [23]. The active gene probe was an 800-base pair HindIII/BamHI fragment from the 5′ end of the beta-major globin gene in pUC 13 [pUC-HB-globin; 16].

### Histone Isolation and Electrophoresis

Whole nuclei, total soluble chromatin and affinity fractionated samples were extracted with 0.25 N HCl at 0°C for 30 min, followed by two 5 sec sonic bursts at a setting of "2." Extracts were centrifuged 10 min at 8,000*g*, and supernatants were dialyzed (3,500 MW cutoff) against three 1.5 liter changes of ice-cold 0.01% SDS, 0.1 mM PMSF, 0.01 mM EDTA, and lyophilized.

SDS polyacrylamide gel electrophoresis was performed essentially as described previously [24]. Samples were resuspended in SDS sample buffer, briefly sonicated, and heated at 100°C for 5 min. Electrophoresis was performed on 22% polyacrylamide SDS gels at 4°C. Gels were impregnated with Fluoro-Hance (RPI), dried, and exposed to Kodak XAR-5 film at −80°C.

### Ubiquitin Western Blotting

After electrophoresis, gels were equilibrated in transfer buffer (20 mM Tris, pH 8.3, 150 mM glycine, 0.01% SDS) for 2 h and electroblotted at 10 V for 16 h onto 0.1 μm nitrocellulose (PH 79, Schleicher & Schuell) that had been thoroughly wetted overnight in DW. Filters were baked at 70°C for 30 min and immunochemically reacted for ubiquitinated proteins as previously de-

scribed [25] except that 50 mg/ml BSA instead of 25 mg/ml BSA was used. Reacted blots were autoradiographed as previously described [24]; bulk protein on the filters was stained for 30 min with Kooh-I-Noor India ink (0.1%) in 50 mM Tris (pH 7.4), 0.03% Tween-20, 150 mM NaCl.

## RESULTS

This procedure to affinity isolate a fraction of active chromatin after nicking active domains with exogenous DNase I [14,15] and repair with Bio-19-SS-dUTP is illustrated in Figure 1. Induced, rather than non-induced, MEL cells were used because the latter contain unacceptably high levels of endogenous nuclease activity that may obscure the specificity of DNase I for active chromatin [16]. Nuclei were nicked with 3.0 units (U)/ml DNase I because this level results in minimal digestion of the active globin gene while providing optimal enrichment of globin gene sequences in the bound affinity fraction [16]. The efficiency of solubilization was judged to be satisfactory, because, after shearing, over 90% of the total chromatin and 80% of the repaired chromatin domains remained in the supernatant following centrifugation at 10,000*g* for 5 min.

Because chromatin was solubilized using relatively harsh physical methods, it was of concern that this may have caused the dissociation of protein from DNA. To estimate the integrity of solubilized chromatin, nuclei from cells prelabeled with $^{14}$C-thymidine and $^{3}$H-lysine + arginine were solubilized and the fragmented chromatin was sedimented through a linear sucrose gradient. As shown in Figure 2, no free protein was observed at the top of the gradient, and the ratio of protein to DNA was similar throughout the gradient. Although this result suggests that the solubilization procedure did not dissociate protein and DNA, this of course does not rule out the possibility that proteins may have been exchanged among the active and inactive chromatin fractions during solubilization.

For affinity chromatography, conditions were established to optimally bind the solubilized, biotinylated chromatin to streptavidin and biotin cellulose. Picomoles of Bio-19-SS-dUTP incorporated during the repair reaction were estimated from the incorporation of $^{32}$P-dCTP, assuming that equimolar quantities of the bio-

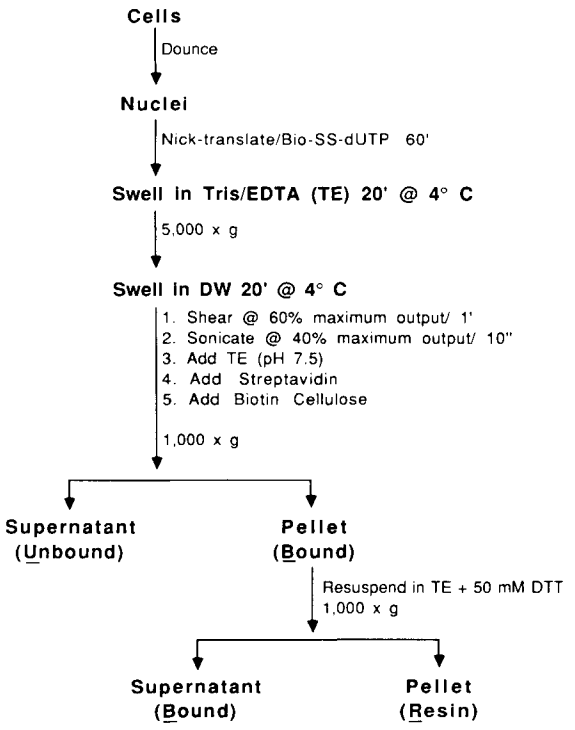


**Fig. 1.** Chromatin fractionation scheme. MEL cell nuclei were affinity labeled by nicking and repair with Bio-19-SS-dUTP, followed by solubilization and fractionation as described in Materials and Methods. To solubilize, affinity labeled nuclei were swollen in TE and centrifuged to produce a globular pellet. The pellet was resuspended in DW, sheared with a Virtis homogenizer, and sonicated. The solubilized chromatin (in TE) was then affinity fractionated with streptavidin and biotin cellulose as detailed in Figure 3.

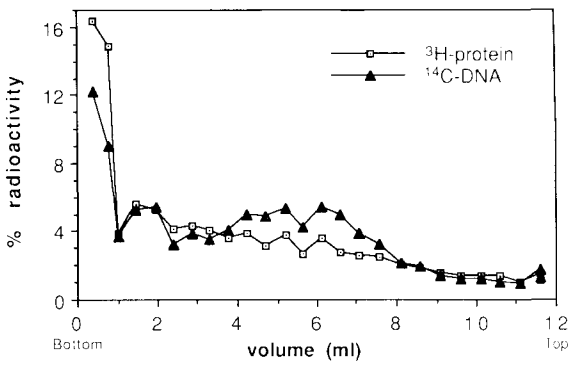


**Fig. 2.** Integrity of solubilized chromatin revealed by association of protein and DNA during sucrose gradient centrifugation. Isolated nuclei from induced MEL cells prelabeled with $^{14}$C-thymidine and $^{3}$H-lysine + arginine were *DNase I-digested,* affinity labeled, and solubilized by shearing and sonication as shown in Figure 1. Solubilized chromatin was applied to a 5–25% linear sucrose gradient and centrifuged for 16 h at 30,000 rpm using a SW41 Ti rotor. Fractions of 0.5 ml were collected from the bottom of the gradient and counted.

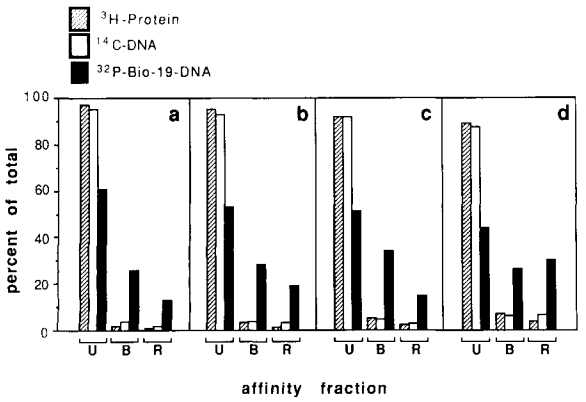


**Fig. 3.** Fractionation of biotinylated, solubilized chromatin with streptavidin (SA) and biotin cellulose (BC). MEL cells were prelabeled with $^{3}$H-lysine + $^{3}$H-arginine and $^{14}$C-thymidine as described in Materials and Methods. Isolated nuclei were nicked with 3.0 units (U)/ml DNase I and repaired with $^{32}$P-dCTP and Bio-19-SS-dUTP; biotin incorporation was estimated from the amount of $^{32}$P-dCTP incorporated. After solubilization, chromatin was fractionated using various picomolar ratios of streptavidin (SA) and biotin cellulose (BC) to Bio-19-SS-dUMP (X) in chromatin: **a,** 10X SA-100X BC; **b,** 20X SA-200X BC; **c,** 25X SA-500X BC; **d,** 25X SA-1000X BC. The first two fractions of flow-through chromatin were pooled as the unbound (U) chromatin. The third and fourth fractions were eluted with 50 mM DTT and pooled as the bound and released (B) fraction. A significant amount of bound chromatin was retained (R). DNA ($^{14}$C-DNA) and protein ($^{3}$H-Protein) in each fraction was expressed as a percentage of the total input TCA precipitable counts. A ratio of 25X SA–1,000X BC (d), which bound the greatest amount of $^{32}$P-dCTP labeled chromatin, was used in subsequent chromatography. Diagonal bars, protein ($^{3}$H-lysine, $^{3}$H-arginine); open bars, DNA ($^{14}$C-thymidine); dark bars, biotin labeled DNA ($^{32}$P-dCTP).

tinylated analog and dCTP were incorporated. Solubilized chromatin was incubated with an excess of streptavidin (SA), followed by a larger excess of biotin cellulose (BC). As shown in Figure 3, various picomolar ratios of SA and BC to incorporated Biotin-19-SS-dUMP (X) were tested to determine optimal binding of biotinylated chromatin. At all ratios, as expected, total bulk protein (diagonal bars; $^{3}$H-protein) was distributed with total bulk DNA (open bars; $^{14}$C-DNA). Most of this nucleoprotein was in the unbound (U) fraction. By contrast, chromatin that was bound and released (B) as well as that bound and retained (R) contained little bulk *nucleoprotein*; however, these fractions were highly enriched in biotinylated chromatin (dark bars; $^{32}$P-Bio-19-DNA). Considering both bound fractions (B and R), maximal binding of approximately 57% of the $^{32}$P-labeled biotinylated chromatin was achieved using a 25-fold excess of streptavidin and a 1,000-fold excess of biotin cellulose to Biotin-19-SS-dUMP in DNA (Fig. 3d).

In our previous study, Southern hybridization of affinity fractionated DNA revealed that enrichment of active globin sequences in the bound and released (B) fraction was approximately 16-fold [16]. To compare this level of enrichment with that of affinity fractionated chromatin, the determination shown in Figure 4 was performed. The ethidium bromide-stained gel (Fig. 4A) revealed that all chromatin fractions contained a similar range of DNA sizes. After blotting and hybridization with a probe [pUC-HB-globin; 16] that recognizes the active beta major globin gene, densitometry of the autoradiograph (Fig. 4B) revealed that, similar to affinity isolated DNA [16], globin sequences in both the bound and released (B) and the bound and retained (R) fractions of chromatin were enriched approximately 15-fold as compared with the unbound (U) fraction. The distribution of hybridized fragments as a smear in Figure 4B was caused by size reduction during shearing and sonication; specificity for the active globin gene was shown by the recognition of the 7.0 kbp EcoRI restriction fragment (arrow, lane $MEL_E$). Hybridization analysis of the same blot shown in Figure 4A with an albumin gene probe (pmalb 2; Fig. 4C) revealed that inactive albumin sequences were distributed uniformly among the active (B,R) and inactive (U) fractions, with a slight enrichment in the unbound (U) fraction.

To characterize the chromatin fractions in terms of ubiquitinated histones, the Western blot determination shown in Figure 5 was performed. Proteins were acid-extracted from each chromatin fraction, separated on a 22% acrylamide-SDS gel, transferred to nitrocellulose, and reacted with anti-ubiquitin antibody. It is noted that the major ubiquitinated histone species, uH2A, co-migrated with the uH2B marker (Fig. 5B); this phenomenon has been repeatedly observed and is under further investigation. The reactivity of the antibody with the core and H1 histones was non-specific, due to the large mass of protein present in these bands. By contrast, uH2A was recognized with great sensitivity (Fig. 5B), as evidenced by the inability to detect stainable uH2A protein (Fig. 5A). From this determination it is concluded that uH2A was evenly distributed among the MEL chromatin fractions.

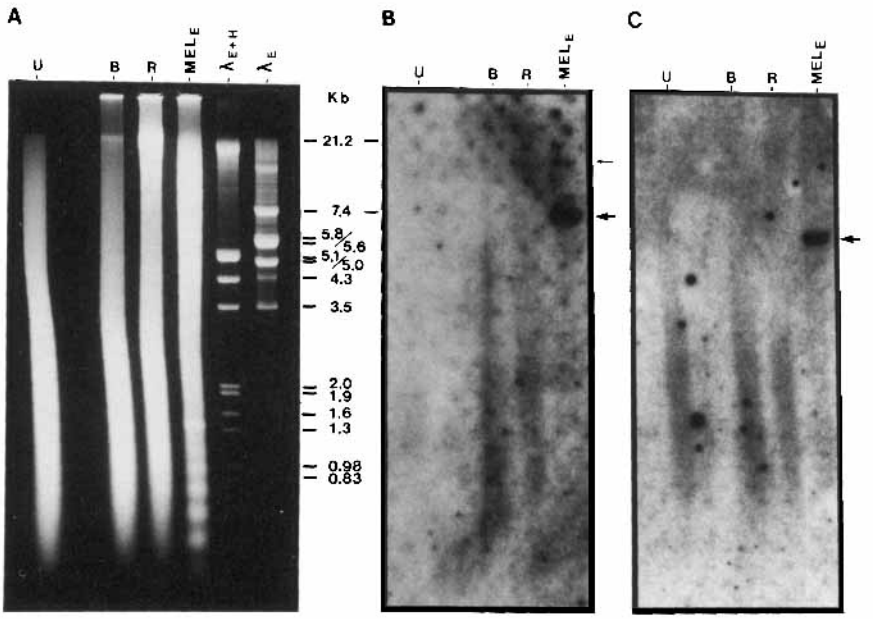


**Fig. 4.** Distribution of active and inactive gene sequences in chromatin fractions. **A:** EtBr-stained 1.0% agarose gel of unbound (U), bound and released (B), and bound and retained (R) chromatin. Five micrograms of DNA from each chromatography fraction were applied to each lane. EcoRI-restricted DNA from MEL cells ($MEL_E$) was included to verify the specificity of the active (pUC-HB-globin) and inactive (pmalb 2) gene probes for restriction fragments containing their respective genes (see **B,C**). Lambda $_{E+H}$ and lambda$_E$ indicate lambda DNA cut with EcoRI + HindIII and EcoRI, respectively, for use as size markers (kilobase pairs, kb). The broad size distribution of DNA fragments in the chromatographed samples was caused by shearing and sonication of chromatin during solubilization. **B:** DNA was alkaline transferred from the gel shown in A to Zeta Probe and hybridized with the active gene probe (pUC-HB-globin) [16] as described in Materials and Methods. Specificity of the probe for the beta major globin gene is shown by the hybridized 7.0 kb EcoRI restriction fragment (large arrow); the small arrow denotes the beta minor globin gene restriction fragment, which is partly complementary to this probe. Note that the bound DNA samples (B,R) contain virtually all of the globin gene fragments; their exhibition as a broad smear presumably resulted from size reduction during shearing and sonication. **C:** The same blot shown in B was stripped and re-hybridized with a probe (pmalb 2) [23] complementary to the albumin gene, which is inactive in these cells. Specificity for the albumin gene is shown by hybridization to the 6.0 kb restriction fragment (arrow). Inactive, albumin sequences are similarly distributed among the bound (B,R) and unbound (U) fractions.

## DISCUSSION

Several affinity approaches have been used to isolate active nucleosomes, including HMG 14-17 agarose and glass bead affinity chromatography [26,27], HMG-17 immunoaffinity chromatography [28,29], and, most recently, Hg-affinity isolation of nucleosomes that contain exposed histone H3 sulfhydryl groups [13]. Unlike these techniques, which select for active chromatin based on the presence or conformation of certain nuclear proteins, the technique described in this paper is based on the well-established specificity of DNase I [14] for active chromatin. Using this approach, we have obtained evidence that uH2A is not localized in transcriptionally active regions of the genome.

A concern in this affinity isolation scheme regards the method employed to solubilize the chromatin. While solubilization must be sufficient to ensure the optimal chromatographic behavior of chromatin particles, it must also be accomplished using conditions that minimize the degradation or exchange or proteins. In these studies, shearing and sonication were necessary to achieve satisfactory solubilization. Less drastic approaches such as digestion of MEL nuclei with a variety of restriction endonucleases (Hind III, EcoRI, Hae III, and Msp I) resulted in solubility of less than 10% of the total chromatin. This was only slightly improved by digestion with micrococcal nuclease; moreover, subsequent lysis of micrococcal nuclease-treated nuclei resulted in the solubilization of less than 50% of the bulk chromatin or the affinity labeled fraction. Thus, physical methods were assessed to improve the extent of solubility. Although sonication of lysed nuclei solubilized only 15% of the bulk or affinity labeled chromatin, the com-

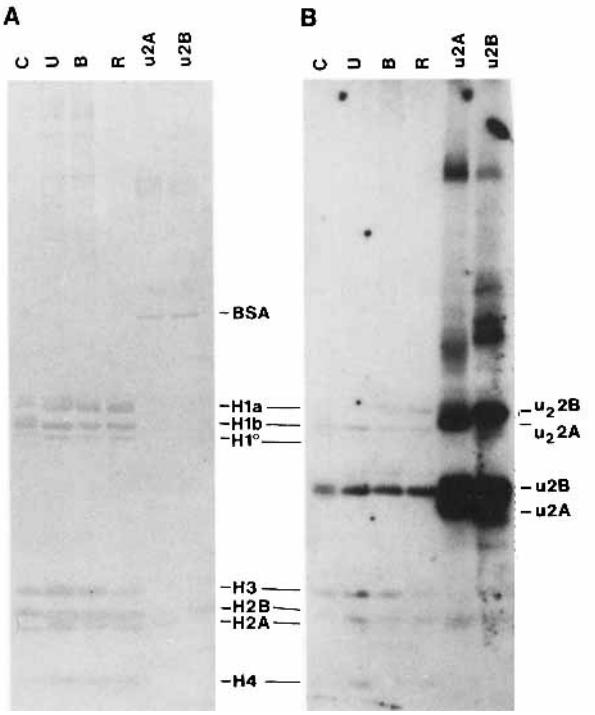


**Fig. 5.** Western blot assessment of ubiquitinated histones in active and inactive chromatin. Equal amounts of $^3$H-labeled protein from unbound (U), bound and released (B), and bound and retained (R) chromatin fractions were separated on a 22% acrylamide/SDS gel. Proteins were electro-blotted onto nitrocellulose and reacted with anti-ubiquitin antibody as described in Materials and Methods. Standard markers included uH2A (**lane u2A**), uH2B (**lane u2B**), and chicken erythrocyte histones (C). **A:** India ink-stained nitrocellulose after Western blotting. **B:** Western blot autoradiograph showing ubiquitinated histones. The signal obtained near the standard $u_2$H2A ($u_2$2A) and $u_2$H2B ($u_2$2B) is due to cross-reactive antibody binding to histone H1s. The content of ubiquitinated H2A was similar in each chromatography fraction.

bination of shearing and sonication resulted in the solubilization of 94% of the bulk and 83% of the affinity labeled chromatin, which was considered adequate for chromatographic separation. It is noteworthy that the affinity fractionation profile of physically solubilized chromatin (Fig. 3) was similar to that previously reported for purified DNA [16].

That protein was not dissociated from DNA during solubilization was indicated by the sucrose gradient analysis (Fig. 2). In addition, the stoichiometry among both core and H1 histones was the same before and after solubilization of total chromatin (data not shown). Furthermore, the protein to DNA ratio was not altered by the chromatographic separation of soluble chromatin into bound (B) and unbound (U) fractions (Fig. 3). However, because these determinations considered protein to DNA and protein to protein stoichiometry only at the level of bulk chromatin, an exchange of histones or other nuclear proteins among chromatin domains cannot be ruled out. One approach to assessing the extent, if any, of protein exchange during solubilization and chromatography would be to use cross-linking methods similar to those used recently to assess the exchange of histones between normal and density-labeled octamers [30].

Efforts to localize ubiquitinated histones in chromatin have resulted in equivocal findings [for review see 18]. Because ubiquitin is released from histone H2A at mitosis, it was initially postulated that ubiquitin ligation prevents nucleosomes from forming compact 30-nm fibers [31]. Indirect evidence for an association of uH2A with active chromatin was that reductions in uH2A were observed at the time of transcriptional shutdown during terminal erythropoiesis [32]; we have reported similar findings during terminal myogenic differentiation [24]. Direct evidence for uH2A localization in active chromatin was shown by its enrichment in nucleosomes containing active *Drosophila* genes [19]. It has also been reported that chicken erythrocyte chromatin that is 50-fold enriched in beta-globin sequences contains slightly increased levels of uH2A and uH2B (1.3- and 3.7-fold enrichment, respectively) [6]. More recently, modest enrichments of ubiquitinated histones in salt-soluble, putatively active chromatin fractions from bovine thymus and chicken erythrocyte nuclei as well as the transcriptionally active *Tetrahymena* macronucleus have been reported [7].

By contrast, others have reported that uH2A is not associated with active chromatin. Using two-dimensional nucleosome mapping, Levinger [33] demonstrated the presence of uH2A in *Drosophila* nucleosomes that contain inactive satellite DNA, while others [21] showed that nucleosomes of the active kappa immunoglobulin light chain gene in mouse plasmacytoma cells are not ubiquitinated. Findings from this laboratory have indicated that although micrococcal nuclease-generated, salt-soluble oligonucleosomes from myotube nuclei are highly enriched in uH2A, they are not co-enriched with these cells' transcriptionally active genes [10]. These findings are consistent with the results in this paper demonstrating that uH2A is randomly distributed in the bound and released (B), bound and retained (R), and unbound (U) fractions of

affinity chromatographed MEL chromatin (Fig. 5).

In summary, although the genomic location of ubiquitinated histones remains controversial, the weight of recent findings indicate a lack of specific involvement with transcriptionally active nucleosomes. Although the role of histone ubiquitination remains unresolved, our recent observations that members of the H2A class, which contains the most extensively ubiquitinated histones, are the most rapidly degraded histones in myotube cells [34] are consistent with the well-established finding that cytoplasmic proteins are ubiquitinated to target them for proteolytic degradation [17].

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