Histone H2A ubiquitination is a bulky posttranslational modification that occurs at the vicinity of the binding site for linker histones in the nucleosome. Therefore, we took several experimental approaches to investigate the role of ubiquitinated H2A (uH2A) in the binding of linker histones. Our results showed that uH2A was present in situ in histone H1-containing nucleosomes. Notably in vitro experiments using nucleosomes reconstituted onto 167-bp random sequence and 208-bp (5 S rRNA gene) DNA fragments showed that ubiquitination of H2A did not prevent binding of histone H1 but it rather enhanced the binding of this histone to the nucleosome. We also showed that ubiquitination of H2A did not affect the positioning of the histone octamer in the nucleosome in either the absence or the presence of linker histones.

Despite the renewed interest in histone H2A/H2B ubiquitination (1–3), the functional role of uH2A still remains controversial. This is in contrast to ubiquitinated H2B where a strong correlation with transcriptional activation has long been established (2), and even a molecular mechanism involving a transhistone regulatory pathway has been shown to be involved in this process (4, 5). With uH2A, there is almost as much experimental evidence for its association to actively transcribing chromatin as there is to the opposite. For instance, it was shown that nucleosomes from the transcriptionally poised mammalian dihydrofolate reductase gene (7) and the transcriptionally active Drosophila hsp70 and copia genes contain 50% uH2A (6). Also nucleosomes containing mono- and diubiquitinated H2A were found to preferentially occur near the 5’-end of the transcribed mouse dihydrofolate reductase gene (7). On the other hand, early studies with rat liver nuclei linked the disappearance of uH2A to increased transcription (8), and it was shown that Drosophila nucleosomes consisting of non-transcribed 1.705 satellite DNA showed an enrichment in uH2A (9). In support of these early data, a role for histone uH2A in Polycomb silencing has been demonstrated recently (10). Furthermore, more uH2A has been shown to be concentrated in the heterochromatin inactive sex body in pachytene spermatocytes (11). Nevertheless the molecular mechanism(s) involved remains completely unknown.

Despite the controversy, ubiquitinated histones have been associated with chromatin partially depleted of linker histones (12) and transcriptionally active or poised sequences (1, 2, 6, 7, 13) that are thought to be have a reduced linker histone content or have altered association with linker histones (for a review, see Ref. 14). However, there is evidence that suggests that histone H2A ubiquitination does not interfere with linker histone binding. Two independent studies have shown that H1 can be cross-linked to uH2A in vitro (15) and in vivo (16). Moreover, in the cross-linking studies performed in mouse cells, the molar ratio of H1-uH2A to H1-H2A was the same as the molar ratio of uH2A to H2A (16).

Linker histones are required for the stabilization of well defined chromatin fibers (17–19), and interference with the binding of linker histones could affect chromatin condensation or the stability of the folded structures. Under physiological conditions, the structure of most linker histones is comprised of three domains: a strongly basic unstructured NH2-terminal tail, a nonpolar globular domain, and another strongly basic unstructured tail at the COOH terminus (20). The three-dimensional structure of the globular domain of histones H1 and H5 has been determined using two-dimensional NMR (21, 22), and the structure of the globular domain of histone H5 was further resolved to 2.5 Å following the determination of its crystal structure (23). Interestingly the three-helix bundle structure of the globular domain of histone H5 has been reported to resemble that of the bacterial catabolite gene activator protein (22, 23) as well as that of the DNA recognition motif of hepatocyte nuclear factor-3 (HNF-3), a Drosophila transcription factor (22). This structural similarity led to a model in which the primary binding site of linker histones to DNA is comprised of helix III of the globular domain binding to the major groove and binding of the β-hairpin to the adjacent minor groove (21, 23).

Despite this, the position of the globular domain of a linker histone on the nucleosome has been quite controversial and may indeed be variable. The issue of the symmetrical or asymmetrical protection of the flanking DNA ends by histone H1 in the chromatosome remains unsettled (24, 25). However, the precise location of the globular domain on the vicinity of the pseudodyad axis is now well established (for reviews, see Refs. 26 and 27).

The COOH terminus of H2A has been found to make contacts with the linker DNA in nucleosomes containing at least 167 bp of associated DNA (28, 29). In the case of core particles lacking linker DNA, the H2A COOH tail repositions to contact the DNA of the dyad axis (29). The H2A COOH tail is therefore...
likely to have some influence on linker histone binding at the nucleosomal level since some DNA contact sites are shared. The crystal structure of the nucleosome (30) indicates that the H2A COOH-terminal tail emerges from the nucleosome near the proposed site of the linker histone globular domain. It has been shown that the contacts between the H2A COOH-terminal tail and the linker DNA are altered slightly in the presence of linker histone (31, 32) indicating that the H2A tail domain is repositioned upon linker histone binding. Ubiquitination of H2A is a bulky modification that occurs at lysine 119 at the beginning of the COOH-terminal tail (1), and it would therefore be expected to interfere with linker histone binding.

In this work, we used a defined in vitro system using individually purified components to investigate the effects of histone ubiquitination on linker histone binding. The results obtained with this system indicate that, contrary to the expectations, ubiquitination of histone H2A did not preclude but rather favored the binding of linker histones to nucleosomes. This observation is in very good agreement with the latest transcriptional silencing role ascribed to this histone H2A modification (10).

MATERIALS AND METHODS

Isolation of Chicken Erythrocyte Nuclei, Nucleosomes, and Core Histones—Chicken nuclei and nucleosome core particles were prepared as described previously (33, 34). Alternatively bulk nucleosomes were isolated from chicken erythrocyte nuclei following a protocol based on the method described by Olins et al. (35). Briefly in this method the nuclear chromatin (at approximately 6 mg/ml of DNA) in 0.1 m KCl, 50 mM Tris-HCl (pH 7.5), 1 mM CaCl2) buffer is subjected to an extensive micrococcal nuclease digestion (150 units of micrococcal nuclease for 24 min at 37 °C), and the whole nuclear digest is then hypotonically lysed by extensive dialysis against 0.25 mM EDTA. The dialysate is next centrifuged at 8,000 × g for 20 min at 4 °C, and the supernatant is dialyzed again against 0.1 m KCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.5) to precipitate the linker histone-containing nucleosomes. Centrifugation under the same conditions as above leads to a supernatant consisting of linker histone-depleted nucleosome fraction and a linker histone fraction in the pellet. Histones from both fractions were extracted with 0.5 m HCl and precipitated overnight at 4 °C with 6 volumes of acetone.

Purification of Linker Histones from Chicken Erythrocyte Nuclei—Extraction of linker histones from chicken erythrocyte nuclei was carried out using CM Sephadex C-25 as described by Garcia Ramirez and colleagues (36). Purified linker histones were stored in 50% (v/v) glycerol at −20 °C and were found to remain stable for up to 1 year. Storage in glycerol counteracts the problem of histone precipitation that occurs upon repeated freezing and thawing of histone samples as reported by Kaplan et al. (37).

Fractionation and Purification of Calf uH2A—uH2A was purified from calf thymus histones with a slight modification of the protocols described elsewhere (38, 39). Briefly 250 mg of 0.4 N HCl-extracted histones were chromatographed at room temperature on a 4.5 × 100-cm BioGel P60 (100–200 mesh) column in 50 mM NaCl and 20 mM HCl buffer (40) at a flow rate of 40 ml/h. H2A fractions from three BioGel P60 column runs were pooled, adjusted to 6 M urea and 50 mM Tris-HCl (pH 7.5), 1 mM CaCl2) buffer is subjected to an extensive micrococcal nuclease digestion (150 units of micrococcal nuclease for 24 min at 37 °C), and the whole nuclear digest is then hypotonically lysed by extensive dialysis against 0.25 mM EDTA. The dialysate is next centrifuged at 8,000 × g for 20 min at 4 °C, and the supernatant is dialyzed again against 0.1 m KCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.5) to precipitate the linker histone-containing nucleosomes. Centrifugation under the same conditions as above leads to a supernatant consisting of linker histone-depleted nucleosome fraction and a linker histone fraction in the pellet. Histones from both fractions were extracted with 0.5 m HCl and precipitated overnight at 4 °C with 6 volumes of acetone.

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Native 4.5% PAGE—Native 4.5% PAGE in 20 mM sodium acetate, 1 mM EDTA, 40 mM Tris-HCl (pH 7.2) buffer for the analysis of nucleosome core particles was carried out according to Yager and van Holde (41).

SDS-PAGE—Electrophoretic analysis of the histones was carried out by SDS-PAGE (20% (w/v) acrylamide, 0.1% (w/v) N,N'-methylenebis(acrylamide)) according to Ref. 42. On occasion (Western blots) the precast NuPAGE Novex bis-Tris gels from Invitrogen were used.

Western Blot Analysis—Western blot analysis was carried out using a rabbit polyclonal antibody prepared in our laboratory. The primary antibody was used at a dilution of 1:1,000, and the dilution of the secondary (goat anti-rabbit) was 1:10,000.

Reconstitution of Histone Octamers—Histone octamers consisting of native H2A or uH2A were reconstituted as described previously (38, 40).

Preparation of 167-bp Random Sequence DNA—Preparation of 167-bp nucleosome cores was carried out as described in Ref. 43. DNA was purified from these nucleosomes by phenol extraction.

Purification of Defined Sequence DNA Templates—pSS 208-12 plasmid containing 12 tandem repeats of a 208-bp sequence derived from the Lytechinus 5 S rRNA gene (44) was prepared as described elsewhere (45). The 208-12 DNA and 208 DNA were excised from the plasmid by HhaI and A vaI digestion, respectively. DNA fragments of interest were separated from the remainder of the plasmid by centrifugation through a linear 5–12% (w/v) sucrose gradient in TE (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) buffer for 1 h at 4 °C at 111,000 × g.

Reconstitution of Nucleosomes—Nucleosome cores were reconstituted on purified 167-bp random sequence or sequence-defined 208-bp 5 S rDNA fragments using the reconstituted hybrid chicken erythrocyte octamers as a histone source containing either calf H2A (control) or calf uH2A and following the procedures already described (45).

Reconstitution of Linker Histones onto Nucleosome Cores—Nucleosomes were reconstituted with linker histones (chicken erythrocyte H1) by either direct addition (25) or by salt dialysis. In this latter instance, reconstituted nucleosome cores and linker histones in 550 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4) were mixed in the ratios stated in the text and dialyzed for at least 4 h against 10 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4).

The reconstituted products were purified by 5–20% (w/v) sucrose
gradient fractionation in 10 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4) at 111,000 g for 16 h at 4 °C, and fractions were analyzed by 0.7% (w/v) agarose gel electrophoresis in 0.5 TBE (45 mM Tris borate, 1 mM EDTA (pH 8.3)) buffer.

DNA Melting Profiles—Reconstituted nucleosome samples at a concentration of 50 g/ml in the buffer specified in the text were heated at a rate of 1 °C/min from 30 to 100 °C. Absorbance at 260 nm was recorded every 0.3 °C using a Pye Unicam SP 1800 spectrophotometer equipped with a custom made temperature controller interfaced to an analog to digital device (Oasis/4, 3D Digital Design and Development Ltd., London, UK) and an IBM 386 computer. Where indicated, the first derivatives of A260 versus temperature profiles were numerically deconvoluted by fitting a sum of Gaussian curves using a Marquardt-Levenberg algorithm.

Determination of Nucleosome and Chromatosome Positioning—The positioning of the histone octamer in the nucleosomes and chromatosomes reconstituted onto 208-bp 5 S rDNA was carried out as described elsewhere (46, 47).

RESULTS

Histone H2A Ubiquitination Does Not Preclude Histone H1 Binding—Nucleosomes consisting of either H2A or uH2A were reconstituted onto random sequence 167-bp DNA, and the binding of histone H1 was assayed. Several unsuccessful attempts were made to reconstitute histone H1 by direct addition (results not shown). The failure of linker histone reconstitution by direct addition was attributed to the histone octamers adopting an equilibrium position on the 167-bp DNA that was unfavorable to successfully form chromatosomes. It has been shown previously that octamer position can affect linker histone binding (48). The short length (≈22 bp) of the linker region available for H1 binding could also contribute to this problem.

Therefore, we decided to use a linker histone reconstitution technique that involves addition of the linker histones to nucleosomes or chromatin at elevated salt concentrations
(0.5–0.6 M NaCl) and subsequent dialysis into low salt buffer (see “Materials and Methods”). At the high salt concentrations, the cationic charges of linker histones are shielded, and ionic interactions with the chromatin DNA are disrupted. If unfavorable octamer positions were inhibiting correct linker histone binding, this reconstitution protocol should allow repositioning of the octamer since it is known that octamers are mobile on DNA (49) especially at higher salt concentrations (50). The histone composition of the chromatosome peaks from the sucrose gradient fractionation following the reconstitution procedure are shown in Fig. 2, A and B. These results showed that uH2A did not prevent linker histone binding to 167-bp nucleosome cores in agreement with previous results (15, 16) and that linker histone binding was slightly favored in the presence of uH2A. The H1 content of uH2A hybrid and control 167-bp cores reached the levels found in a total acid extract of calf thymus at a molar input ratio of 1 and 1.3 histone H1/mol of nucleosome core (as above) were analyzed by thermal denaturation (Fig. 4). Under the conditions used, all samples exhibited a triphasic hyperchromicity curve. The first two transitions are thought to be associated with the melting of the DNA associated with the ends of the nucleosome core, while protein denaturation and melting of the DNA more tightly associated with core histones represent the main transition (51, 52). In 167-bp control and uH2A hybrid nucleosome cores, the first transition occurred at 69 and 68 °C and represented 13% (21 bp) and 15% (25 bp) of the DNA, respectively. This could reflect a slightly decreased interaction of histone tails with the ends of the nucleosome core DNA in the presence of uH2A. This decrease in protein-DNA interaction was more subtle than that caused by core histone acetylation where a decrease of 3 °C in the Tm of the first transition was observed (53). In the presence of linker histones, the first transition decreased in magnitude...
Denaturation was carried out at 50°C to 70°C for both control and uH2A hybrid nucleosomes. This decrease in magnitude of the first transition was slightly more pronounced for the uH2A hybrid nucleosomes than for control chromatosomes. The association of linker histones with the ends of the core DNA would be expected to stabilize this DNA, correlating with the observed increase in the $T_m$ value of the first transition of 1°C and 2°C for control and uH2A hybrid chromatosomes, respectively. The second and third transitions of both samples occurred at 79°C and 83.8°C, respectively, indicating that the presence of uH2A did not lead to large scale disruption of octamer-DNA interactions in good agreement with previous observations (38, 54).

Histone H1 Equally Affects the Positioning of the Histone Octamer in the Chromatosome Regardless of the Ubiquitinated Nature of Histone H2A—We decided next to determine the effect of ubiquitination of H2A on the position of chromatosomes on 5S rDNA. To this end, chicken histone H1 was reconstituted with control and uH2A hybrid nucleosomes by direct addition at 50 mM NaCl (25). As described above, this methodology was not suitable for reconstituting linker histones onto 167-bp nucleosome cores. However, direct addition of linker histones to 208-bp nucleosomes resulted in the formation of distinct chromatosome bands (see Fig. 5), suggesting that the additional linker DNA plays a role during linker histone binding.

To determine the affinity of the interaction between histone H1 and control and uH2A hybrid nucleosomes, the nucleosome binding.

Histone H1 and control and uH2A hybrid nucleosomes, the nucleosome binding.

Histone H1 and control and uH2A hybrid nucleosomes, the nucleosome binding.

To confirm linker histone binding and to determine the influence of uH2A on chromatosome positioning, control and uH2A 208-bp nucleosomes were reconstituted with a molar input ratio of 0.75 histone H1/nucleosome and digested with micrococcal nuclease. Very definite pauses at 167 bp, characteristic of linker histone protection of DNA (55, 56), were observed in both cases from the trial digests (not shown). Although kinetic stops at 167 bp are observed in nucleosomes (43, 57, 58), comparison of trial digests of nucleosomes reconstituted in the absence of H1 and chromatosomes demonstrated that 167-bp bands would no longer have been present in nucleosomes lacking linker histones at the optimal digestion time used to produce 167-bp bands from chromatosomes. Furthermore 208-bp uH2A or H2A reconstituted nucleosomes produced, under similar digestion conditions, only a 151-bp nucleosomal band. Fig. 6, A and B, shows the results obtained when the 167-bp bands were excised and digested with several restriction enzymes as described under “Materials and Methods.” The results were essentially identical for control and uH2A hybrid nucleosomes. In the presence of histone H1, a dominant position (-4 to 163) was observed that is occupied by ~80% of chromatosomes (Fig. 6B, see position A). This can be envisaged to correspond to a 10-bp protection of the DNA on either side of position A or a 20-bp protection downstream of position B (see Fig. 6B). This position and the shift from several other minor translationally related positions (such as that shown in Fig. 6B, position C) are in close agreement with a major position of chromatosomes on n-mer arrays of 208 template DNA (47, 59). A 10-bp pattern of protection on either side of the nucleosome (Fig. 6B, main position A) agrees with earlier data (55, 56, 60, 61). Under the experimental conditions used by us, no evidence could be found for asymmetric protection of linker DNA as has been observed with Xenopus 5S rDNA (25, 62, 63). Interestingly, this chromatosome position in Xenopus 5S RNA has been found more recently to be susceptible to sequence-specific artifacts, and another major chromatosome position that involves protection of 20 bp on one side of the nucleosome has also been reported (24, 64, 65). If this type of protection were the case here, it implies that the incorporation of histone H1 led to a shift in the equilibrium position of nucleosomes from position A to position B. Alternatively, since nucleosomes are more mobile in the absence of linker histones (66), it is possible that digestion with micrococcal nuclease could induce short range sliding of nucleosomes.

Since positions A and B are -10 bp apart, the rotational setting of the DNA relative to the histone octamer would be unchanged. A summary of nucleosome and chromatosome positions is shown in Fig. 6B.

**DISCUSSION**

Although the functional role of histone H2A ubiquitination still remains quite obscure, a strong correlation has been established over the years between this posttranslational modification and transcriptionally active chromatin (1–3). Transcriptionally active chromatin has long been associated to linker histone depletion (14). Hence it was of interest to deter-
mine the structural constraints imposed by H2A ubiquitination and histone H1 on each other at the nucleosome level.

The results shown in Fig. 1 as well as other previously published data (15, 16) clearly show that the occurrence of H2A ubiquitination can take place indistinctively in linker histone-containing nucleosomes and chromatin. The in vitro studies conducted here on the binding of histone H1 to nucleosomes consisting of 167-bp random sequence or 208-bp sequence-defined (5 S rRNA) DNA (Fig. 3) are fully consistent with these findings.

The results of this study are highly reminiscent of those obtained with acetylated histones, another posttranslational modification of histones that has been strongly correlated with chromatin transcriptional activity (67–69). Similarly to what has been described here, it was found that core histone acetylation did not block the binding of linker histones to nucleosomes (70) and that this modification did not have any major
influence on either the mobility of the histone octamers or the positioning of the nucleosomes (71).

Our observations about the enhanced histone H1 binding to uH2A-containing nucleosomes are helpful in addressing the still controversial functional role of this histone H2A modification. They clearly support the early implications of a repressive

FIG. 6. A, determination of chromatosome positioning on the 208-bp 5 S rDNA template. Shown is 8% polyacrylamide electrophoresis of restriction enzyme cleavage products of chromatosome DNA isolated after micrococcal nuclease digestion of chromatosomes containing either uH2A or H2A. Lane M contains pBR 322/HpaII size markers. Lane U contains undigested chromatosome 167-bp DNA. Lanes A, D, and S contain DNA fragments produced by digestion of chromatosome DNA with AvaI, DraI, and Sau3AI, respectively. *, a 232-bp fragment from plasmid pGBT9 lacking AvaI, DraI, and Sau3AI restriction sites was included as an internal control. B, summary of control and uH2A hybrid nucleosome and chromatosome positions on the 208-bp *Lytechinus* 5 S rDNA fragment. Dominant positions are shown in **bold**.
role for uH2A (8, 9, 11) as well as its very recent involvement in Polycomb silencing (10). As we pointed out, the mechanisms by which uH2A contributes to PcG silencing are not known. In this regard, the higher histone H1 binding affinity for uH2A nucleosomes would clearly validate this in vivo observation while providing a potential contributing structural component of the molecular mechanism(s) involved.

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