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Structural Changes of Nucleosomal Particles and Isolated Core-Histone Octamers Induced by Chemical Modification of Lysine Residues[†]

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ABSTRACT: Treatment of nucleosomal particles and isolated core-histone octamers with dimethylmaleic anhydride, but not with acetic anhydride, is accompanied by a biphasic release of the two H2A·H2B dimers, the first dimer being more easily released than the second. With both kinds of particles, 50% of histones H2A and H2B are released for modification of approximately 35% of the histone amino groups. The similar behavior of nucleosomal particles and isolated core-histone octamers is consistent with the same structure of the histone octamer in the nucleosomal particle and in the free octamer in 2 M NaCl. The described release of H2A·H2B dimers allows the preparation of nucleosomal particles deficient in one H2A·H2B dimer and of the histone hexamers H2A·H2B·(H3·H4)$_2$. For more extensive modifications, both reagents, acetic and dimethylmaleic anhydrides, cause the dissociation of nucleosomal particles with liberation of double-stranded DNA, which suggests that lysine amino groups are involved in the binding of histones to DNA. The modified nucleosomal particles are more sensitive to ionic strength than those untreated, and the presence of salt (NaCl) increases the extent of DNA release. The histones corresponding to the liberated DNA, except H2A and H2B released with dimethylmaleic anhydride, are apparently bound to the DNA-containing particles as extra histones.

The forces that bind double-stranded DNA to the core-histone octamer to form nucleosomal particles appear to be essentially electrostatic. Changes in these electrostatic interactions, as those produced by the physiological acetylation of lysine residues, might cause structural alterations of functional significance (Weisbrod, 1982; Reeves, 1984). The electrostatic forces within the nucleosomal particle are weakened by increasing the ionic strength and by modification of the interacting charged groups. Acylation of lysine residues has been used to change the interaction of histones with DNA. The chemical acetylation of chromatin decreases the salt concentration needed to release core histones (Wong & Marushige, 1976). Treatment of nucleosomal particles with dimethylmaleic anhydride causes rearrangement of the nucleosomal components, with release of histones H2A and H2B and of single-stranded DNA, and formation of residual particles deficient in histones H2A and H2B but containing an excess of H3 and H4 (Jordano et al., 1984a,c). Structural studies of the residual particles are consistent with the stabilization by histones H2A and H2B of a DNA length of 50–70 base pairs per nucleosome (Jordano et al., 1984b). Regeneration of the modified amino groups of the residual particles plus the complementary fraction containing histones H2A and H2B is accompanied by reconstitution of nucleosomal particles with the structural properties of the original nucleosomes (Jordano et al., 1984a,b). Recently, we have

found that the release of single-stranded DNA during modification is caused by the local and transient changes in pH induced by the base added to prevent the fall in pH (Nieto & Palacián, 1987). When modification takes place in the absence of base addition, no release of single-stranded DNA is observed.

The present work describes the structural changes of nucleosomal particles and core-histone octamers produced by modification of lysine residues with acetic and dimethylmaleic anhydrides, in the absence of base addition, as well as the effects of moderate salt concentrations on the modified nucleosomal particles.

MATERIALS AND METHODS

Preparation of Nucleosomal Particles and Histone Octamers. Nuclei were obtained from chicken erythrocytes after lysis of the cells in a buffer solution containing Nonidet P40 (Weintraub et al., 1975). The isolated nuclei were digested with micrococcal nuclease (Cooper) and extracted with 0.25 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0) (Lacy & Axel, 1975). Nucleosomal particles containing histones H1 and H5 were precipitated from the extract with 100 mM KCl, resuspended in 10 mM Tris-HCl (pH 8.2) containing 5 mM EDTA, and dialyzed overnight at 4 °C against the same buffer solution. From the dialyzed preparation, a mononucleosomal fraction was isolated by centrifugation in 5–20% sucrose gradients containing the resuspension buffer components. To dissociate histones H1 and H5, the mononucleosomal preparation was dialyzed against 10 mM Tris-HCl (pH 8.2), 0.7 M NaCl, and 5 mM EDTA. The nucleosomal particles were separated from the released histones by sucrose gradient centrifugation in the presence of 0.7 M NaCl. DNA from nucleosomal particles had an average length of 160 base pairs, as determined by polyacrylamide gel electrophoresis, with the digestion products of plasmid pBR322 treated with nuclease *Hae*III used as length markers. Core-histone octamers were prepared from nuclei by salt extraction (4 M NaCl) after removal of histones H1 and H5 (0.7 M NaCl), as described by Ruiz-Carrillo and Jorcano (1979), except that for the extraction 4 M NaCl was used instead of 2 M NaCl.

Chemical Modification of Nucleosomal Particles and Histone Octamers. Nucleosomal particles (0.1 mg of DNA/mL) and histone octamers (5 mg/mL), in 200 mM N-[tris(hydroxymethyl)methyl]glycine (Tricine) (pH 8.2) and variable NaCl concentration (2 M for histone octamers), were treated at room temperature with different amounts of acetic anhydride (AA) or dimethylmaleic anhydride (DMMA) (dissolved in dioxane, 50–200 mg of anhydride/mL). Reaction took place in the absence of base addition. The modified preparations were dialyzed at 4 °C against 10 mM Tris-HCl (pH 8.2), 5 mM EDTA, and variable NaCl concentration (2 M for histone octamers).

Determination of the Modification Degree. The extent of modification of protein amino groups was determined in two different ways: by measuring the radioactivity incorporated into the macromolecules from ^{14}C-labeled AA or by evaluating the extent of reaction with trinitrobenzenesulfonic acid of the modified preparations. The radioactivity incorporation procedure is suitable for determining the degree of modification of protein amino groups in nucleosomal particles, because the modifications by carboxylic acid anhydrides of the DNA moiety and that of other protein reactive groups are negligible (Bernad et al., 1986). With this procedure, the extent of modification produced by DMMA was evaluated from the radioactivity incorporated in the DMMA-modified preparation when treated with ^{14}C-labeled AA, under conditions that cause
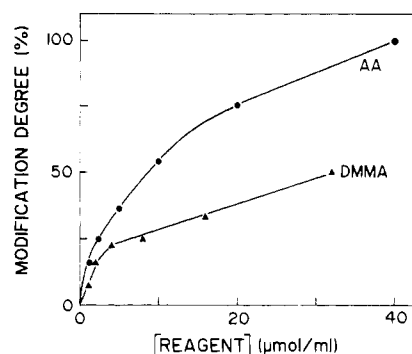

FIGURE 1: Degree of modification of nucleosomal particles treated with different amounts of AA and DMMA. Nucleosomal particles were treated, in the absence of NaCl, with the indicated amounts of AA or DMMA. The modification degree was determined by measuring radioactivity incorporated, as described under Materials and Methods.

complete modification of histone free amino groups (treatment with 2 mg of AA/mL, with addition of 1 M NaOH to avoid the fall in pH, not shown).

Analytical Procedures. Preparations were centrifuged in linear 5–20% sucrose gradients containing 10 mM Tris-HCl (pH 8.2), 5 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and the indicated NaCl concentration. Centrifugation took place in a Beckman SW40 rotor at 30 000 rpm and 6 °C for 26 h, for nucleosomal particles, and in a SW65 rotor at 60 000 rpm and 4 °C for 40 h, for histone octamers. The distribution along the gradient of materials absorbing at 254 nm (nucleosomal particles and DNA) or at 280 nm (histones) was determined with an ISCO density fractionator, which was also used for the separation of fractions. Electrophoretic analysis of histones was conducted in polyacrylamide gels, 15% polyacrylamide or a linear gradient from 10 to 20% (Figure 2), containing 0.1% sodium dodecyl sulfate (Laemmli, 1970). Histone concentration was determined according to Bradford (1976) or to Lowry et al. (1951). The extent of DNA dissociated from nucleosomal particles was estimated from the areas corresponding to free DNA and to nucleosomal particles in the sedimentation patterns. The double-stranded nature of DNA was verified by elution from hydroxyapatite columns as previously described (Nieto & Palacián, 1987). Electrophoresis of nucleosomal particles under nondenaturing conditions was conducted in 5% polyacrylamide gels, using as electrophoresis buffer 89 mM Tris–borate, 89 mM boric acid, and 2 mM EDTA.

RESULTS

Figure 1 shows the degree of modification of the amino groups of histones in nucleosomal particles treated with different amounts of AA or DMMA. The extent of modification obtained with AA is much higher than that corresponding to DMMA for equivalent amounts of reagent. Modification is accompanied by a decrease in the sedimentation rate of the particles, in agreement with previous work (Jordano et al., 1984a). For the same degree of modification, DMMA causes a larger change in sedimentation rate than AA (not shown). This result is probably due to the larger expansion of the nucleosomal particles induced by the introduction of negative charges by DMMA and also to the release of histones H2A and H2B by this reagent (see below).

Biphasic Release of H2A·H2B Dimers from Nucleosomal Particles and from the Isolated Core-Histone Octamers by Modification with DMMA. Modification of nucleosomal particles with DMMA causes the release of histones H2A and H2B. Figure 2 shows the sedimentation patterns of prepa-
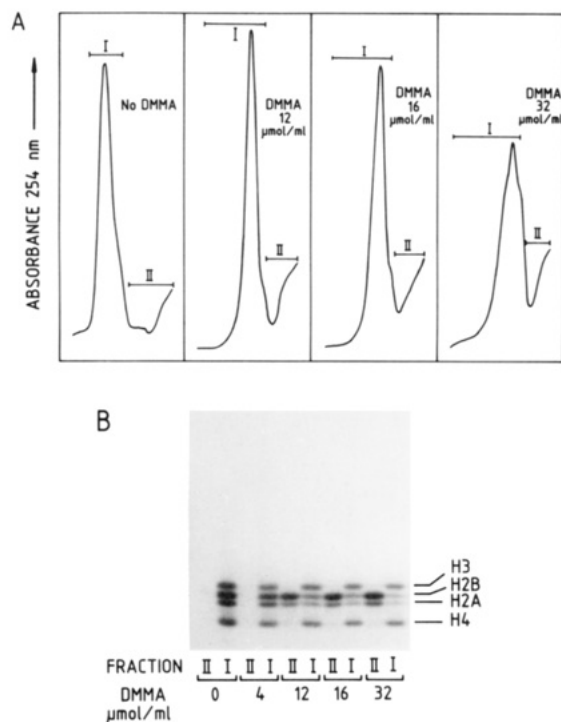
FIGURE 2: Release from nucleosomal particles of histones H2A and H2B by modification with DMMA. Nucleosomal particles were treated, in the absence of NaCl, with the indicated amounts of DMMA. (A) Sedimentation patterns. The bars (I and II) indicate the fractions isolated for histone electrophoresis and determination of the extent of histone release (Figure 4). (B) Electrophoresis of the histones present in the residual particles (fractions I) and of those released by the treatments (fractions II). The amounts of fractions I and II used for electrophoresis were not complementary.

rations modified with different amounts of reagent and the electrophoresis of the histones present in the DNA-containing particles (fractions I) and in the gradient region close to the meniscus (fractions II). The released fractions contain histones H2A and H2B, while the residual particles retain the whole complement of histones H3 and H4 but are increasingly deficient in H2A and H2B. The shoulder in the sedimentation pattern of the preparations treated with 16 and 32 μmol of DMMA/mL (Figure 2A) corresponds to free DNA.

Since the octamer of core histones can be obtained in 2 M NaCl, we wanted to know whether the modification of this particle with DMMA is also accompanied by release of histones H2A and H2B. Sedimentation analysis in sucrose gradients of preparations of histone octamers modified with different amounts of reagent (Figure 3A) shows the formation of a component (II) with a sedimentation coefficient lower than that of the octamer and corresponding to H2A·H2B dimers. In addition, the sedimentation coefficient of the fastest component (I), initially the intact unmodified octamer, decreases with increasing levels of modification. The slower component contains equimolecular amounts of histones H2A and H2B, while the faster component includes all histones H3 and H4 present and decreasing amounts of H2A and H2B.

The release of histones H2A and H2B from nucleosomal particles and histone octamers is very similar, in spite of the very different conditions used for modification (200 mM Tricine for nucleosomal particles and 200 mM Tricine plus 2 M NaCl for histone octamers). The plot of the amount of histones H2A and H2B released versus the amount of reagent used or the degree of modification (Figure 4) gives similar curves for the two kinds of particles. The results also indicate that the degree of modification obtained with a certain amount of reagent is approximately the same, within the experimental
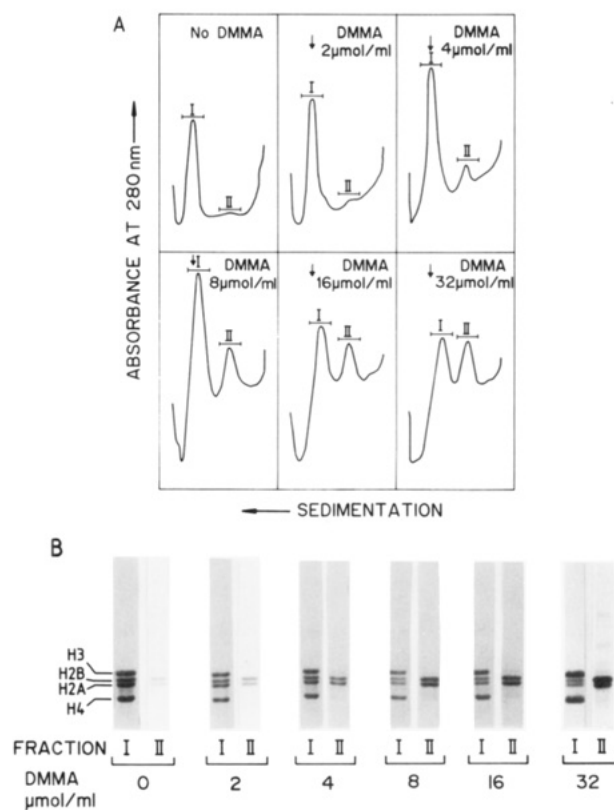

FIGURE 3: Release from histone octamers of H2A·H2B dimers by modification with DMMA. The preparation of histone octamers was treated, in the presence of 2 M NaCl, with the indicated amounts of DMMA. (A) Sedimentation patterns. The bars (I and II) indicate the fractions isolated for electrophoresis and determination of the extent of histone release (Figure 4). The arrows show the position of the unmodified histone octamers. The increase in the total absorbance of the preparation at 280 nm with the amount of reagent employed is due to the contribution of the reagent moieties of the modified amino groups, while the elevation of the base line close to the meniscus is produced by the partial regeneration of the modified groups and release of dimethylmaleic acid during centrifugation. (B) Electrophoresis of the histones present in the fractions isolated by centrifugation. The amounts of fractions I and II used for electrophoresis were not complementary.
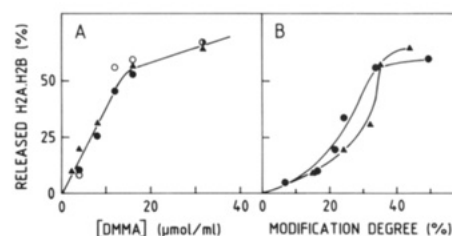

FIGURE 4: Biphasic release of H2A·H2B dimers from nucleosomal particles (●, ○) and from the isolated histone octamers (▲) by modification with DMMA. (A) The amount of histones H2A and H2B released from the particles was determined by measuring the protein content of the fractions isolated by centrifugation (Figures 2 and 3), according to the method of Bradford (1976) (nucleosomal particles, ●) or Lowry et al. (1951) (histone octamers, ▲). With nucleosomal particles, the extent of release of H2A and H2B was also determined by densitometric analysis of fractions I in the gel shown in Figure 2B (○). (B) The data for histone octamers (▲) correspond to the experiment of Figure 3, while those for nucleosomal particles (●) were obtained in an experiment similar to that shown in Figure 2. The modification degree was determined colorimetrically with trinitrobenzenesulfonic acid, for histone octamers, and by measuring radioactivity incorporated, for nucleosomal particles, as described under Materials and Methods.

range studied, for nucleosomal particles and histone octamers. The biphasic release of histones H2A and H2B found in both cases, with the first phase ending after release of approximately
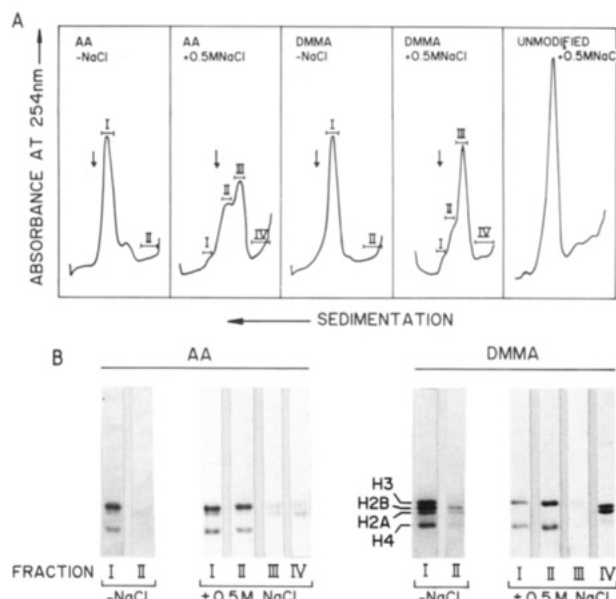
FIGURE 5: Effect of salt (NaCl) on nucleosomal particles modified by AA and DMMA. Nucleosomal particles were treated, in the absence of NaCl, with AA (10 μmol/mL) or DMMA (8 μmol/mL). After modification, the preparations were dialyzed and centrifuged in the absence or presence of 0.5 M NaCl. (A) Sedimentation patterns. The bars (I–IV) indicate the fractions isolated for electrophoresis. The arrows show the position of the corresponding unmodified particles, which were dialyzed and centrifuged under the same conditions as those of the modified particles. (B) Electrophoresis of the histones present in the different fractions isolated by centrifugation.

50% of histones H2A and H2B, for a modification of 35% of the amino groups of histones, and the equimolecular proportion in which both histones are liberated (Figures 2 and 3) are consistent with an asymmetric release of the two H2A·H2B dimers.

In contrast with the results obtained with DMMA, modification of nucleosomal particles (see below) or core-histone octamers (not shown) with AA is not accompanied by liberation of histones H2A and H2B.

Effect of Salt (NaCl) on the Disassembly of Nucleosomal Particles Modified with AA and DMMA. The effect of salt (NaCl) on the disassembly of nucleosomal particles has been studied in two different ways. The nucleosomal particles modified in the absence of salt were treated with different salt concentrations, or the nucleosomal particles were modified in the presence of salt and the salt was eliminated prior to characterization of the modified preparations.

Figure 5 shows the effect of 0.5 M NaCl on nucleosomal preparations previously modified with AA or DMMA in the absence of salt. Without salt addition, only the preparation modified with AA has a small amount of free double-stranded DNA. However, after incubation with 0.5 M NaCl, most of the DNA in both modified preparations is found as free DNA (Figure 5A, component III), and two nucleoprotein components can be distinguished in the sedimentation patterns (Figure 5A, components I and II in the salt-treated preparations). Incubation of unmodified nucleosomal particles in 0.5 M NaCl produces liberation of only a small amount of free DNA (Figure 5A). Salt treatment of the DMMA-modified preparation causes complete release of histones H2A and H2B, while the two nucleoprotein particles, which lack H2A and H2B, retain all the H3 and H4 present in the preparation (Figure 5B).

When modification takes place in the presence of salt and this is eliminated immediately afterward, the results shown in Figure 6 are obtained. The sedimentation patterns of the
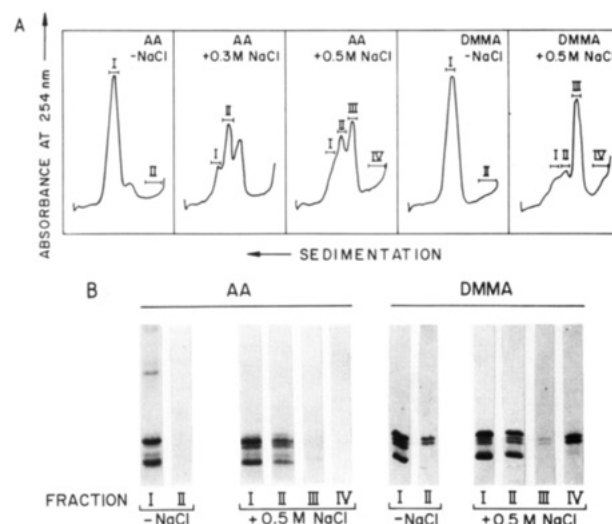

FIGURE 6: Effect of salt (NaCl) during modification of nucleosomal particles with AA and DMMA. Nucleosomal particles were treated with AA (10 μmol/mL) or DMMA (8 μmol/mL), in the absence or in the presence of the indicated concentrations of NaCl. After modification, the preparations were dialyzed and centrifuged in the absence of NaCl. (A) Sedimentation patterns. The bars (I–IV) indicate the fractions isolated for electrophoresis. (B) Electrophoresis of the histones present in the different fractions isolated by centrifugation.
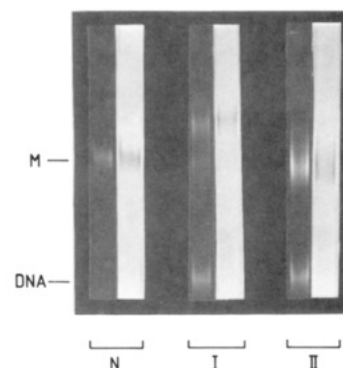

FIGURE 7: Nondenaturant electrophoresis of the two different residual particles obtained by modification of nucleosomal particles with AA in the presence of salt. The samples used were control unmodified nucleosomal particles (N) and the two fractions separated by centrifugation from a preparation modified with AA (10 μmol/mL) in the presence of 0.3 M NaCl (Figure 6A). For each sample, the electrophoresis strip was stained with ethidium bromide (left) and with Coomassie blue (right). The positions of mononucleosomal particles (M) and of nucleosomal-sized DNA (DNA) are shown.

modified preparations are qualitatively similar to those corresponding to preparations modified in the absence of salt and subsequently incubated with salt (Figure 5). However, in this case, the nucleoprotein components in the preparation modified with DMMA in the presence of 0.5 M NaCl are not completely devoid of histones H2A and H2B. Electrophoresis under nondenaturing conditions of the nucleoprotein components present in the preparation modified with AA in the presence of 0.3 M NaCl (Figure 7) shows that fraction II moves as a single component in the position corresponding to untreated mononucleosomal particles, while fraction I gives two well-separated bands. Fraction II and the fast-moving band from fraction I seem to correspond to nucleosomal particles containing one complement of histones and the slow-moving band in fraction I to nucleoprotein particles with two core-histone octamers.

Disassembly of nucleosomal particles with release of free DNA takes place to a low extent in the absence of NaCl (in
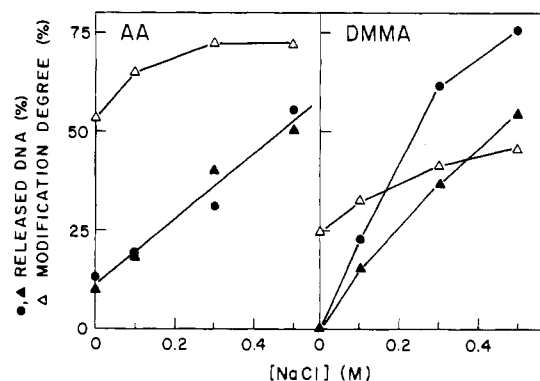
FIGURE 8: Dependence of the amount of DNA released from nucleosomal particles on the salt (NaCl) concentration present during or after modification. Nucleosomal particles were treated with AA (10 μmol/mL) or DMMA (8 μmol/mL). Modification was conducted in the absence of salt (NaCl), and the modified preparation was subsequently dialyzed and centrifuged in the presence of the indicated NaCl concentrations (●), or modification was performed in the presence of the indicated concentrations of NaCl and the subsequent dialysis and centrifugation in the absence of NaCl (▲). In the first case, the degree of modification of the amino groups of histones was 53% for AA and 25% for DMMA, while in the second case, it changed with the salt concentration present during modification, the different values being indicated in the figure (△).

200 mM Tricine). However, the extent of release of free DNA is notably increased by the presence of salt, either during or after modification. Figure 8 shows the results obtained under both experimental conditions. For the preparations modified with AA (10 μmol/mL), the liberation of DNA is approximately the same whether NaCl is present during or after modification. In contrast, with DMMA the extent of DNA release is larger when salt is present after modification. These results indicate that the dissociation of the modified nucleosomal particles by salt is partially reversible on removal of salt for the particles modified by DMMA but not for those obtained with AA. For the preparations modified with AA, 12% of the DNA is released in the absence of salt, while no free DNA is found in the DMMA-modified preparations, which is probably due to the much lower modification degree of the preparations modified with DMMA (25% compared with 53% for the AA-modified preparations). However, for larger amounts of reagent, liberation by DMMA of free DNA is also obtained in the absence of salt (see Figure 2A).

DISCUSSION

The observed biphasic release of equimolecular amounts of histones H2A and H2B from nucleosomal particles and from the isolated core-histone octamers, by modification with DMMA, is consistent with the asymmetric dissociation from the particles of the two H2A·H2B dimers. The response to DMMA treatment is similar for the two kinds of particles. Both the modification degree and the amount of H2A and H2B liberated for a certain DMMA treatment are similar for the two particles, in spite of the very different ionic conditions used during modification (200 mM Tricine, for nucleosomal particles, and 200 mM Tricine plus 2 M NaCl, for histone octamers). These results are in agreement with the proposed structural similarity of the free histone octamer in 2 M NaCl and the histone octamer associated to DNA in the nucleosomal particle (Thomas & Kornberg, 1975; Uberbacher et al., 1986). Apparently, the weakening of the electrostatic repulsion forces brought about by high ionic strength is structurally equivalent to the neutralization of the positive charges of histones by interaction with DNA.

The results reported in a previous work (Jordano et al.,

1985) indicate that the asymmetric dissociation of H2A·H2B dimers induced by DMMA modification is consistent with equivalent binding sites in the nucleosomal particles for the two H2A·H2B dimers rather than with intrinsically different sites. The negative cooperativitiy in the dissociation of the H2A·H2B dimers might be related to the structural dynamics of nucleosomes in vivo. Baer and Rhodes (1983) found that the nucleosomal particles that bind to RNA polymerase II in vitro are enriched in transcribed DNA sequences and are deficient in histones H2A and H2B. They proposed that the nucleosomal particles present in transcriptionally active chromatin may be deficient in H2A and H2B in the cell. Recent work from our laboratory (González et al., 1987) shows that core particles lacking one H2A·H2B dimer interact with RNA polymerase from Escherichia coli more strongly than whole core particles, being also more efficient as transcription templates. A negative cooperativity in the dissociation of the two H2A·H2B dimers from nucleosomal particles would facilitate the formation of particles containing only one H2A·H2B dimer. The finding that, in the cell, newly synthesized H2A and H2B are incorporated as H2A·H2B dimers into nucleosomal particles containing one old (H3·H4)$_2$ tetramer and one old H2A·H2B dimer (Jackson, 1987) suggests that nucleosomal particles deficient in one H2A·H2B dimer are important structural intermediates in chromatin dynamics.

The liberation of H2A·H2B dimers from nucleosomal particles and free core-histone octamers described in this paper allows the preparation of nucleosomal particles lacking one H2A·H2B dimer and of core-histone hexamers formed by one (H3·H4)$_2$ tetramer and one H2A·H2B dimer. Under the modification conditions employed in the present work, release of 50% of the H2A and H2B from a preparation of nucleosomal particles can be obtained without liberation of free DNA, thus avoiding the formation of particles containing extra histones, which takes place when there is liberation of free DNA by the presence of salt (this work) or by addition of base to prevent the fall in pH during modification (Jordano et al., 1984a, 1985).

The liberation of free double-stranded DNA by modification of nucleosomal particles with AA, at salt concentrations much lower than those needed to dissociate untreated nucleosomes (Figure 8), supports the involvement of lysine residues in the binding of histones to DNA in the nucleosomal particle. A similar release of DNA upon modification with AA is also found with trypsin-digested core particles (unpublished results).

Salt concentrations lower than 0.5 M NaCl, which are unable to dissociate native nucleosomal particles, cause the release of free DNA from nucleosomal particles modified with AA or DMMA. In the modified particles, the electrostatic interactions between histones and DNA are already weakened by the modification of lysine residues. Therefore, a small additional effect of ionic strength is sufficient to dissociate DNA from histones.

When modification of nucleosomal particles takes place under conditions that cause liberation of free double-stranded DNA, the histones corresponding to the released DNA bind as extra histones to the residual DNA-containing particles. These results are in agreement with the reported binding of additional molecules of H3 and H4 or core-histone octamers to the surface of nucleosomal cores (Klevan et al., 1978; Voordouw & Eisenberg, 1978; Stein, 1979; Eisenberg & Felsenfeld, 1981). With AA, which causes no dissociation of the core-histone octamer, complete histone octamers would bind to the undissociated nucleosomal particles, while, with DMMA, which causes liberation of H2A·H2B dimers,

(H3·H4)$_2$H2A·H2B hexamers and (H3·H4)$_2$ tetramers would also bind as extra histones, the relative amounts of the three particles depending on the degree of modification and the salt concentration.

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Synthesis of a Biological Active Tumor Growth Factor from the Predicted DNA Sequence of Shope Fibroma Virus[†]

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ABSTRACT: A 55-residue peptide comprising the carboxyl portion (residues 26–80) of the Shope fibroma virus growth factor (SFGF), a predicted 80-residue DNA virus gene product that encoded a homologous sequence with the epidermal growth factor transforming growth factor α family, was synthesized by a stepwise solid-phase method. The synthetic SFGF (26–80) purified to homogeneity by reverse-phase HPLC was characterized by fission ionization mass spectrometry and amino acid analysis. The disulfide pairings were established by enzymatic digestion and mass spectrometry and were found to be similar to those of EGF and TGFα. Synthetic SFGF (26–80) was found to share about 10% of the activities as EGF in the radioreceptor binding to A431 cells, stimulation of [³H]thymidine uptake in NRK cells, and induction of colony formation in soft-agar assay. Our results therefore confirmed that SFGF contained the putative biological activities of the EGF–TGFα family and that production of SFGF by Shope fibroma virus infected cells may account for the proliferative diseases associated with this particular virus.

Poxvirus comprises a large group of eukaryotic DNA virus whose exceptionally large double-stranded DNA genomes replicate within virosomes in the cytoplasm of the infected host cells. Several poxviruses are known to be responsible for proliferative tumorigenic diseases. Three notable examples of tumorigenic poxviruses are the following: (1) Shope fibroma virus (SFV)[1] (Shope, 1932; Chang et al., 1987), which induces benign fibromas in adult rabbit; (2) Yaba tumor virus, which causes subcutaneous histiocytomas in monkeys and man (Bearcroft & Jamieson, 1958); and (3) Molluscum contagiosum, which produces tumor-like epidermal lesions in man

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[1] Abbreviations: Boc, *tert*-butoxycarbonyl; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMS, dimethyl sulfide; EGF, epidermal growth factor; HPLC, high-performance liquid chromatography; SFGF, Shope fibroma virus growth factor; SFV, Shope fibroma virus; TFA, trifluoroacetic acid; TGFα, transforming growth factor α; VGF, vaccinia virus growth factor.