Reconstitution of nucleosomes containing dinitrophenylated histones

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Histone octamers of purified monomer nucleosomes were labelled with [^3H]dinitrofluorobenzene. Authentic 11S nucleosomes were reconstituted *in vitro* from a mixture of [^3H]dinitrophenylated histones and excess unlabelled monomer nucleosomes. The reconstituted nucleosomes were found to contain [^3H]dinitrophenylated histones H2a and H2b but not [^3H]dinitrophenylated histones H3 and H4. Approx. 83% of [^3H]dinitrophenylated nucleosomes were immunoprecipitable with anti-dinitrophenyl immunoglobulin and *Staphylococcus aureus*. These results demonstrate that histones H2a and H2b contain dinitrofluorobenzene-reactive groups that can be modified without destroying their ability to participate in nucleosome formation *in vitro*.

The nucleosome is the basic, repeating structural unit of eucaryotic chromatin. It is composed of approx. 200 base pairs of DNA and an octamer of histones (McGhee & Felsenfeld, 1980). Two different sets of strong histone–histone interactions exist within the histone octamer. The arginine-rich histones, H3 and H4, interact to form a tetramer which plays a fundamental role in the organization of DNA into a nucleosome (Camerini-Otero *et al.*, 1976) and is thought to be involved in the initial assembly of newly-replicated DNA into nucleosomes at DNA replication forks (Worcel *et al.*, 1978; Jackson & Chalkley, 1981). The lysine-rich histones, H2a and H2b, interact to form a H2a–H2b dimer which then completes the nucleosome structure. Newly synthesized histones H2a and H2b are not specifically deposited on newly replicated DNA (Jackson & Chalkley, 1981) and have recently been shown to exchange with nucleosomal histones H2a and H2b (Louters & Chalkley, 1984).

To characterize further the process of nucleosome assembly, we have investigated the ability of histones modified with Dnp-F to interact with DNA to reconstitute nucleosomes *in vitro*. In addition, we have asked if the reconstituted, dinitrophenylated nucleosomes can be specifically immunoprecipitated by anti-dinitrophenyl immunoglobulin.

Abbreviations used: Dnp, dinitrophenyl; Dnp-F, 1-fluoro-2,4-dinitrobenzene; SDS, sodium dodecyl sulphate.

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Materials and methods

Labelling with [^3H]dinitrofluorobenzene

Salt-extracted histones (Ruiz-Carrillo & Jorcano, 1979) or histone octamers (Shaw *et al.*, 1975; Tatchell & Van Holde, 1977) were labelled with 1 μCi of 1-fluoro-2,4-dinitro[3,5- ^3H]benzene (26 Ci/mmol; Amersham International)/ μg of protein as described in the text. The reaction was followed over 24 h at 37°C. Duplicate samples were precipitated with 20% (w/v) trichloroacetic acid in the presence of 20 μg of unlabelled histone. The precipitate was collected by centrifugation, solubilized with 0.5 ml of 1M-NaOH, neutralized with HCl, and [^3H]Dnp-labelled histone present was measured by liquid scintillation methods.

SDS/polyacrylamide-gel electrophoresis

Analysis of [^3H]Dnp-labelled histones on SDS/polyacrylamide gels (18%) was performed as described by Thomas & Kornberg (1980). Each sample contained 3×10^5 c.p.m. of labelled histone and 20 μg of nonlabelled histone. Gels were run at a constant current of 17.5 mA for 16 h and prepared for fluorography as described by Bonner & Laskey (1974).

Nucleosome reconstitution

Following labelling of histone octamers with [^3H]Dnp-F in 2M-NaCl, the reaction was added to a 200-fold excess of nonlabelled monomer nucleosomes. Additional NaCl was added to a final concentration of 2M. The reconstitution was then dialysed in a stepwise fashion against 1.5M, 1.0M,

0.6M- and finally 0.05M-NaCl in 10mM-Tris/HCl (pH 7.4)/0.1mM-EDTA (Tatchell & Van Holde, 1977). The reconstituted nucleosomes were characterized by sedimentation in isokinetic 5–20% neutral sucrose gradients run in an SW41 rotor at 35000 rev./min for 17 h at 4°C. Control ^3H -labelled nucleosomes were reconstituted in an analogous way with histones labelled by reductive methylation using NaB^3H_4 (Tack *et al.*, 1980).

Immunoprecipitation of ^3H Dnp-labelled histones and nucleosomes

^3H Histones, ^3H nucleosomes, ^3H Dnp-labelled histones and ^3H Dnp-labelled nucleosomes were all treated in the same manner, according to the method of Kessler (1975). Briefly, 1 μg of labelled protein was incubated with or without 70 μg of rabbit anti-Dnp immunoglobulin (Winkelhake & Nicolson, 1976) for 3 h at 4°C in Dulbecco's phosphate-buffered saline, pH 7.2. An excess of thoroughly washed Cowan I strain *Staphylococcus aureus* was added and incubation was continued for 1 h at 4°C. The amount of ^3H protein present in the supernatants and pellet was determined.

Results and discussion

Dnp-F reacts with thiol groups, imidazole groups, both α - and ϵ -amino groups and phenolic hydroxyl groups. The reaction proceeds via a bimolecular nucleophilic displacement ($\text{S}_{\text{N}}2$) mechanism involving an unprotonated nucleophile. In our initial experiments, salt-extracted core histones were labelled with ^3H Dnp-F under conditions of increasing pH (Fig. 1*a*). As expected, the extent of labelling increased as the pH of the reactions was increased from 7.0 to 9.5. Conditions for maximum labelling of salt-extracted core histones with ^3H Dnp-F were 0.4M-sodium borate (pH 9.5)/0.05M-NaCl. Under these conditions, histones with a specific radioactivity of 4×10^5 c.p.m./ μg of protein were obtained. At pH 9.5, thiol and imidazole functional groups will react with ^3H Dnp-F. However, since only a single cysteine residue is found in chicken erythrocyte core histones (residue 110 of histone H3), the modifications of core histones that are observed under these conditions most likely involve the imidazole groups of histidine residues. At pH 9.5, both α - and ϵ -amino groups are partially protonated (Hirs, 1967) and are therefore only partially reactive. The reaction was found to proceed slowly, requiring 8 h at 37°C for complete reaction. The buffering capacity of 0.04M-sodium borate was necessary to achieve this level of labelling. When the sodium borate concentration was decreased to 0.1M, pH 9.5, the reaction proceeded to 64% of that shown in Fig. 1*a*.

Subsequent attempts to reconstitute nucleosomes containing histones labelled with ^3H Dnp-F under the above conditions were unsuccessful. We therefore investigated alternative labelling conditions that would maintain good levels of incorporation of ^3H Dnp-F into histones while at the same time minimizing the effect of the derivatization on the ability of the histones to interact with other histones and DNA to form nucleosomes. In 2M-NaCl, monomer nucleosomes dissociate into 145 base pair DNA fragments and histone octamers (Eickbush & Moudrianakis, 1978). We therefore investigated the labelling of the histone octamer produced under these conditions in the hope that critical histone-histone interactions would be preserved during the reaction. The extent of labelling of the histone component of purified, monomer nucleosomes in 0.4M-sodium borate, pH 9.5, in the presence of either 0.05M-NaCl or 2M-NaCl is shown in Fig. 1*b*. The maximum extent of incorporation of ^3H Dnp-F into histones present in intact monomer nucleosomes in 0.05M-NaCl (1.0×10^5 c.p.m./ μg) was approx. 30% of that observed for salt-extracted core histones under the same conditions. The extent of labelling was increased to approx. 50% of that observed for salt-extracted histones when the nucleosome was dissociated into DNA and histone octamers in 2M-NaCl. The dissociation of the DNA from the surface of the histone octamer presumably exposes additional Dnp-F-reactive groups previously protected from the reagent in the intact nucleosome.

To determine if the ^3H Dnp-labelled histone octamers could interact with DNA to form nucleosomes, ^3H Dnp-labelled histone octamers were added to a 200-fold excess of unlabelled, monomer nucleosomes, adjusted to 2M-NaCl and subsequently dialysed in a stepwise fashion into 0.05M-NaCl (Tatchell & Van Holde, 1977). The reconstituted mixture was then characterized by velocity sedimentation in a 5–20% neutral sucrose gradient (Fig. 2). Of the ^3H Dnp-labelled histones applied to the gradient, 70% sedimented as a well-defined peak characteristic of an 11S nucleosome (Fig. 2). This fact was confirmed by sedimentation of authentic monomer nucleosomes on a parallel gradient (results not shown). In addition, the ^3H Dnp-labelled histones cosedimented with the excess, unlabelled nucleosomes that were formed during the reconstitution reaction and detected by A_{260} .

Gradient fractions containing the reconstituted ^3H Dnp-labelled nucleosomes were pooled and their histone composition examined by SDS gel electrophoresis followed by fluorography. While the initial reaction of the histone octamers with ^3H Dnp-F resulted in the labelling of all four core

Fig. 1. Incorporation of $[^3\text{H}]\text{Dnp-F}$ into salt extracted histones and histone octamers (a) Salt-extracted histones were reacted with $[^3\text{H}]\text{Dnp-F}$ as described in phosphate-buffered saline at pH 7.0 (Δ) 0.4M-sodium borate at pH 8.0 ($\square$) and 0.4M-sodium borate at pH 9.5 ($\circ$). (b) Purified monomer nucleosomes were reacted with $[^3\text{H}]\text{Dnp-F}$ in 0.4M-sodium borate, pH 9.5, supplemented with 0.05M-NaCl ($\bullet$) or 2.0M-NaCl ($\circ$). Samples of each reaction were precipitated with 20% (w/v) trichloroacetic acid and counted by standard liquid-scintillation procedures.

Fig. 2. Sedimentation analysis from reconstituted nucleosomes

Nucleosomes were reconstituted from $[^3\text{H}]\text{Dnp}$ -labelled histone octamers and excess, non-labelled nucleosomes and sedimented in 5–20% neutral sucrose gradients. Non-labelled, reconstituted nucleosomes were detected by measuring the A_{260} of each fraction. $[^3\text{H}]\text{Dnp}$ -labelled histones were detected by precipitation of a sample of each fraction as described. The arrow indicates the position of authentic monomer nucleosomes (11S) sedimented in an identical gradient. Sedimentation was from right to left.

histones (Fig. 3, lane b), only $[^3\text{H}]\text{Dnp}$ -labelled histones H2a and H2b were found in the reconstituted nucleosomes (Fig. 3, lane c). $[^3\text{H}]\text{Dnp}$ -labelled histones H3 and H4 appear to be incapable of participating in nucleosome reconstitution *in vitro*. It should be noted that, during the initial labelling reaction, histones H2a and H2b were more extensively labelled than were histones H3 and H4. As much as 80% of the total $[^3\text{H}]\text{Dnp}$ groups incorporated into histone octamers can be attributed to histones H2a and H2b. Nevertheless, if $[^3\text{H}]\text{Dnp}$ -labelled histones H3 and H4 had been reconstituted into nucleosomes with the same efficiency as histones H2a and H2b, their presence would have been detectable. This result suggests that the derivatization of histones H3 and H4 with $[^3\text{H}]\text{Dnp-F}$ results in a structural alteration which precludes their subsequent involvement in nucleosome formation. We therefore conclude that the $[^3\text{H}]\text{Dnp}$ -labelled reconstituted nucleosomes are composed of unlabelled H3–H4 histone tetramers derived from the excess unlabelled nucleosomes in the reconstitution mixture, and $[^3\text{H}]\text{Dnp}$ -labelled histones H2a and H2b. The sequential interaction of H3–H4 tetramers followed by H2a–H2b dimers with DNA during nucleosome reconstitution *in vitro* has been noted by others (Louters & Chalkley, 1984; Ruiz-Carrillo & Jorcano, 1979).

Fig. 3. Fluorograph of an SDS/18% polyacrylamide gel containing salt-extracted histones labelled by reductive methylation with NaB^3H_4 (a), histones from monomer nucleosomes labelled with $[^3\text{H}]\text{Dnp-F}$ in 2 M-NaCl (b) and $[^3\text{H}]\text{Dnp}$ -labelled histones present in reconstituted nucleosomes (c).

Table 1. Specific immunoprecipitation of $[^3\text{H}]\text{Dnp}$ -labelled histones and $[^3\text{H}]\text{Dnp}$ -labelled monomer nucleosomes

Sample	Anti-Dnp immunoglobulin present	Radioactivity in pellet (%)
$[^3\text{H}]\text{Histone}$	—	0.8
	+	0.6
$[^3\text{H}]\text{Nucleosome}$	—	5.5
	+	4.1
$[^3\text{H}]\text{Dnp-labelled histone}$	—	5.8
	+	80.3
$[^3\text{H}]\text{Dnp-labelled nucleosome}$	—	8.7
	+	82.7

Reconstituted $[^3\text{H}]\text{Dnp}$ -labelled nucleosomes were purified by sedimentation in sucrose gradients as described above and immunoprecipitated with anti-Dnp immunoglobulin and *S. aureus*. As a control, nucleosomes reconstituted in the presence of histones labelled by a reductive methylation with NaB^3H_4 were used. As shown in Table 1, both $[^3\text{H}]\text{Dnp}$ -labelled histones and $[^3\text{H}]\text{Dnp}$ -labelled nucleosomes were specifically immunoprecipitated. Of the dinitrophenylated histones, 80% were immunoprecipitated by the anti-Dnp immunoglobulin whereas only 5.8% was non-

specifically adsorbed by *S. aureus*. A similar fraction (82.7%) of the dinitrophenylated histones present in $[^3\text{H}]\text{Dnp}$ -labelled nucleosomes was immunoprecipitated. These results indicate that, even though the DNA is wrapped around the surface of a histone octamer in a nucleosome, its presence does not prevent the recognition of $[^3\text{H}]\text{Dnp}$ -labelled histones H2a and H2b by anti-Dnp immunoglobulin.

The ability to immunoprecipitate selectively nucleosomes containing dinitrophenylated histones H2a and H2b may provide a useful approach to the isolation of newly assembled nucleosomes from chromatin replication systems *in vitro*.

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