Disruption of Reconstituted Nucleosomes

THE EFFECT OF PARTICLE CONCENTRATION, MgCl₂, AND KCl CONCENTRATION, THE HISTONE TAILS, AND TEMPERATURE*

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We find that reconstituted nucleosome cores containing specific DNA sequences dissociate on dilution. This disruption of histone-DNA contacts leading to the release of free DNA is facilitated by the presence of the core histone tails, MgCl₂ (5 mM), KCl (60 mM), and temperatures above 0 °C. Under reaction conditions that are commonly used to assess transcription-factor access to nucleosomal DNA, histone-DNA contacts are on the threshold of instability. We demonstrate how dilution of reconstituted nucleosomes containing a TATA box can facilitate TBP access to DNA.

The nucleosome core is a fragile object (van Holde, 1988). Systematic study of the stability of nucleosome cores isolated from the nuclei of somatic cells has determined the temperature, pH, and salt concentrations at which histone-DNA interactions are disrupted (Zama et al., 1979; Liberti and Small, 1980; Burch and Martinson, 1980; Burton et al., 1978; Walker and Wolffe, 1984). An important aspect of nucleosome core stability is the sensitivity of histone-DNA interactions to dilution. The fraction of intact nucleosome cores decreases as the total nucleosome concentration is lowered (Stacks and Schumaker, 1979; Lilley et al., 1979; Cotton and Hamkalo, 1981; Eisenberg and Felsenfeld, 1981; Yager and van Holde, 1984; Ausio et al., 1984a, 1984b). This dissociation of nucleosome cores into histones and free DNA under dilute conditions is substantial at physiological ionic strengths. Cotton and Hamkalo (1981) found that more than 20% of the nucleosome cores would dissociate at a concentration of 10 ng/μl at physiological ionic strength over a 2-h period. Lilley et al. (1979) in determining the consequences for nucleosome integrity of association with eukaryotic RNA polymerase II found that “at the low nucleosome concentrations used to achieve enzyme excess for nucleosome transcription experiments, dissociation to free DNA is considerable, irrespective of the presence of polymerase.”

More recent work has made extensive application of nucleosome cores reconstituted using defined sequences of DNA (Archér et al., 1991; Chen et al., 1994; Côté et al., 1994; Hayes and Wolffe, 1992; Imbalzano et al., 1994; Kwon et al., 1994; Lee et al., 1993; Li et al., 1994; Li and Wrangle, 1993; Perlmann and Wrangle, 1988; Pina et al., 1990; Workman and Kingston, 1992). Radiolabeling of the DNA used in these experiments has facilitated the use of very dilute solutions. In certain instances nucleosome disruption directed by trans-acting factors has been documented (Chen et al., 1994; Côté et al., 1994; Imbalzano et al., 1994; Kwon et al., 1994; Workman and Kingston, 1992). Most of these experiments make use of reconstituted nucleosomes under dilute conditions ranging from 6 ng/μl (Workman and Kingston, 1992) to 0.1 ng/μl (Imbalzano et al., 1994). Thus it is possible that spontaneous nucleosome disruption might influence the outcome of these experiments.

We have examined the integrity of nucleosome cores reconstituted so as to contain the Xenopus borealis 5 S RNA gene. DNA sequences of this type are among those with the highest affinity for the histone octamer (Shrader and Crothers, 1989; Schild et al., 1993). The X. borealis 5 S RNA gene also directs the positioning of the histone octamer with respect to DNA sequence, offering the opportunity to examine the consequences of nucleosome dissociation on the DNase I cleavage of DNA within a positioned nucleosome core (Rhodes, 1985; Hayes et al., 1990). We find that the 5 S nucleosome and other reconstituted nucleosomes dissociate on dilution under the standard binding conditions for transcription factors such as TBP.

MATERIALS AND METHODS

DNA Constructs—Unless otherwise noted, all experiments were performed with a modified Xenopus 5 S RNA gene in which the adenovirus major late promoter TATA box (TATAAAG) replaces native sequence from −73 to −66 relative to the start site of 5 S RNA gene transcription (+13; see Pruss et al. (1995)). This replacement was obtained by polymerase chain reaction mutagenesis of plasmid XP-10 (Wolffe et al. (1986). The construct was digested with EcoRI and RsaI to liberate a 154-bp fragment. For Fig. 5 the following constructs were used: a 159-bp DNA fragment that was amplified using polymerase chain reaction from the Xenopus TR/Jα gene (from +322 to +362 relative to the start site of transcription at +1 (Runyan et al., 1994)) and a 424-bp DNA fragment obtained by digestion of a plasmid containing two tandem repeats of the X. borealis somatic 5 S RNA gene (psX5S197–2) (Ura et al., 1995). All DNA fragments were end-labeled with T4 polynucleotide kinase or Klenow fragment (New England Biolabs) and gel purified.

Transcription Factors—Recombinant Saccharomyces cerevisiae TBP (Hoffman and Roeder, 1993; Harikashi et al., 1989) was expressed in and purified from Escherichia coli as described (Nikolov et al., 1992) except that trypsin cleavage of the histidine-tag was omitted. Recombinant S. cerevisiae TFIIA was a kind gift of Dr. Yoshioiro Nakatani at NIH.

Nucleosome Reconstitution—Nucleosome core particles were purified from chicken erythrocyte nuclei as described previously (Wolffe and Hayes, 1993). Trypsinized octamers were prepared as described by Wolffe and Hayes (1993).

Dilution Conditions—Unless noted, standard buffer conditions for TBP/TFIIA binding to DNA were used throughout. Reconstituted nucleosome core particles were diluted to the desired concentrations in TE (10 mM Tris, pH 8.0, 1 mM EDTA) in a volume of 7.2 μl before being mixed with the following buffer in 25 μl reactions. The final buffer concentration after mixing was 20 mM HEPES/KOH, pH 7.8, 5.6 mM dithiothreitol, 12 mM Tris·HCl, pH 7.8, 3 mM MgCl₂, 60 mM KCl, 6% glycerol, 60 μg/ml bovine serum albumin, and 1 μg/ml dGdC. As indicated, both MgCl₂ and KCl concentrations were varied for certain experiments while leaving the other buffer conditions unchanged. Mock incubations (as would be required for TBP binding) were typically performed at 30 °C for 30 min. The temperature of these incubations was also varied as indicated.

Unless otherwise noted, electrophoresis was in 0.7% agarose and 0.5 × TBE (1 × TBE is 90 mM acid, 2.5 mM EDTA) for 3 h at 100 V.

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DNase I Footprinting—Reconstituted nucleosome core particles were spun on a 5–20% sucrose gradient in TE plus 1 mM phenylmethylsulfonyl fluoride (20 h at 35,000 rpm in a Beckman SW 41 rotor) to remove unreconstituted DNA. These were treated as described above, scaling up 7-fold. Following incubation, samples were digested with DNase I at a concentration of 12 µg/ml for 5 min at room temperature. The reaction was stopped with a 2-fold excess of EDTA, made 0.25% SDS and 0.3 M NaAc, and extracted with phenol/chloroform and chloroform alone prior to precipitation. Electrophoresis was on a 6% denaturing gel (Hayes et al., 1993). The different bands can then be assayed for phosphorylation.

### RESULTS AND DISCUSSION

Nucleosome Disruption in Vitro

Nucleosome Core Disruption Facilitates TBP Binding—We first examined the stability of nucleosome cores that contained a modified 5 S rRNA gene into which the adenovirus major late TATA box (TATATAAAG) had been inserted (see “Materials and Methods”). The TATA box was positioned 81 bp from the dyad axis of the nucleosome core (Pruss et al., 1995). Control digestions with DNase I and micrococcal nuclease, which determine the rotational and translational position of DNA relative to the histone octamer, confirmed that these were unchanged from those obtained with the wild type X. borealis 5 S rRNA gene (see Fig. 2). Our binding conditions were optimized to facilitate the association of yeast TBP and TFIIA with the TATA box in naked DNA (Fig. 1, lane 2) (Horikoshi et al., 1989, 1992; Buratowski et al., 1989; Maldonado et al., 1990). Ionic conditions were 20 mM HEPES/KOH, pH 7.8, 12 mM Tris-HCl, pH 7.8, 5.6 mM dithiothreitol, 60 µM bovine serum albumin, 1 µM dGdC, 3 mM MgCl₂, 60 mM KCl, and 6% glycerol. Addition of reconstituted nucleosome cores (see “Materials and Methods”) to this solution at progressively lower concentration leads to the progressive dissociation of the nucleosomes as revealed by native polyacrylamide gel electrophoresis (Fig. 1, lanes 3–5). Note that because of dilution a smaller mass of DNA and hence radioactivity is loaded in lane 5 compared with lane 3. At 3 ng/µl, histone octamer-associated DNA exceeds free DNA by ~3-fold (determined from the phosphorimager), whereas at 1 ng/µl, free DNA exceeds histone octamer-bound DNA by more than 2-fold (Table I). Thus the relative proportions of nucleosomal compared with free DNA change significantly simply through adjustment of concentration. Control experiments indicated that DNA was not being lost through nonspecific association with the reaction tube. These involve measuring the low levels of radioactivity associated with the reaction tube after the sample had been loaded on the gel or most simply by removal of the solution from the tube following the dilution process and measuring any residual radioactivity in the tube by scintillation counting.

We next examined the binding of TBP and TFIIA to free and nucleosomal DNA. The TBP/TFIIA proteins bind to the TATA box within naked DNA but not to the TATA box when it is associated with an unmodified octamer of histones (Fig. 1, lanes 6–8). Incubation of nucleosomes with TBP/TFIIA under progressively more dilute conditions leads to a progressively larger proportion of TBP/TFIIA bound to naked DNA appearing with dilution (Fig. 1, lanes 6–8, Table I). This is because histone-DNA contacts are selectively disrupted on dilution. Thus a potential contributory factor in the access of TBP to nucleosomal DNA (Imbalzano et al., 1994) could be the dissociation of nucleosomes at high dilution. Imbalzano et al. (1994) use reconstituted nucleosomes at a very low concentration of 0.1 ng/µl. As a control for the capacity of our gel system to resolve a tertiary complex of histones, TBP/TFIIA, and DNA, we examined the binding of TBP/TFIIA to nucleosomal particles from which the core histone tails had been removed using trypsin (”tail-less nucleosomes”). Earlier work had indicated that the major impediment to TFIIA access to DNA in a nucleosome containing the X. borealis 5 S rRNA gene was the interaction of the core histone tails with DNA (Lee et al., 1993). TBP/TFIIA efficiently forms a tertiary complex with the tail-less nucleosome containing the TATA box (Fig. 1, lanes 9 and 10). Thus the core histone tails impede TBP/TFIIA access to nucleosomal DNA under these conditions.

A concern in this type of analysis is that gel electrophoresis might introduce a potential artifact. Nucleosomes might dissociate upon entry into the gel. An experiment that might verify or eliminate any gel dissociation artifact is to carry out a DNase I footprinting cleavage, followed immediately by separation into free DNA and nucleosomes on a non-denaturing gel (Wolffe and Hayes, 1993). The different bands can then be assayed for cleavage pattern after elution and deproteinization in a denaturing gel. If the “free DNA” is really free, then it should show no 10–11-bp periodicity in cleavage. Such a 10–11-bp periodicity in cleavage might reflect a nucleosomal organization in solution that is lost on electrophoresis. We carried out this experiment using nucleosomal DNA in dilute solution (1 ng/µl) and found that “free DNA” in the gel was cleaved by DNase I without any 10–11-bp periodicity, i.e. it is digested as naked DNA (Fig. 2A, lane 1). In contrast, nucleosomal DNA isolated from the native gel showed a clear 10–11-bp periodicity of cleavage (Fig. 2A, lane 2). This result suggests that nucleosomes do not dissociate during electrophoresis under our experimental conditions. It should also be noted that there is little smearing of nucleosomal DNA, reflecting the continued stability of the complex once it has entered the gel matrix.

A feature of nucleosomal disruption by the yeast or human SWI/SNF complexes is the loss of DNase I cleavage patterns characteristic of the nucleosome (Côté et al., 1994; Imbalzano et al., 1994). Consistent with earlier data on mixed sequence nucleosomes (Ausio et al., 1984a, 1984b) and the 5 S nucleosome containing a TATA box (Fig. 1), dilution of nucleosomes will contribute to their disruption. In fact, DNase I cleavage of reconstituted nucleosomes incubated under progressively more
Dilute conditions lead to the progressive loss of protection from DNase I cleavage (Fig. 2, lanes 2–4). In the earlier published experiments (Côté et al., 1994; Imbalzano et al., 1994) nucleosomes are not isolated from native gels following DNase I cleavage (Wolffe and Hayes, 1993); thus mixed populations of free DNA and histone-bound DNA might complicate interpretation of the experimental results. Moreover, the efficiency of reconstitution into nucleosomes might vary with DNA template; for example Shrader and Crothers (1989) report wide variation in the stability of DNA-histone interactions. Without examining nucleoprotein complexes on native gels or by analytical ultracentrifugation it is almost impossible to determine reconstitution efficiencies.

We suggest that it is important to control for nucleosome dissociation under the dilution conditions used in in vitro experiments when assessing trans-acting factor access to nucleosomal DNA or the disruption of nucleosomes by molecular complexes such as SWI/SNF.

The Influence of the Histone Tails, Ionic Conditions, and Temperature on Nucleosome Dissociation—Rigorous earlier work had made use of analytical ultracentrifugation to determine the thermodynamic parameters governing nucleosome dissociation using nucleosome core particles containing a mixture of DNA sequences (Ausio et al., 1984a, 1984b). Ausio and colleagues also presented a thermodynamic analysis of the existing data on this topic (Stacks and Schumaker, 1979; Cotton and Hamkalo, 1981; Ausio et al., 1984a, 1984b). Our results are in general agreement with this careful analysis. Here we have also examined more qualitative aspects of nucleosome dissociation using reconstituted nucleosome cores to which a second histone octamer is bound (Ausio et al., 1984a, 1984b).

**Fig. 2.** DNase I cleavage of naked DNA and nucleosomal DNA isolated from native gels and of disrupted nucleosomes in solution. A, sucrose gradient-purified core particles were diluted to 1 ng/μl before digestion with DNase I, and nucleoprotein complexes were then resolved on a non-denaturing gel before elution, deproteinization, and resolution on a denaturing gel. The digestion pattern of “free DNA” (lane 1) and nucleosomal DNA (lane 2) is shown. Arrowheads indicate the 10–11-bp pattern of DNase I cleavage in nucleosomal DNA. B, sucrose gradient-purified core particles at the indicated concentrations (per μl) after cleavage with DNase I. MgCl₂ concentration is 7 mM. Lane 1 contains G-specific cleavage reaction as a marker, lanes 2-4 contain diluted core particles, and lane 5 is naked DNA. The 10–11-bp periodicity indicative of a rotationally phased nucleosome is evident in lane 2 but becomes progressively more like naked DNA in lanes 3 and 4.

**Fig. 3.** Effect of histone tails on nucleosome disruption. A, the stability of nucleosome core particles with histone tails removed by trypsin compared with non-treated core particles upon dilution. MgCl₂ concentration is 3 mM. B, effect of MgCl₂ concentration on tail-less octamer stability. Core particles with histone tails removed incubated with different levels of MgCl₂. Lanes 1–5 contain no MgCl₂, and lanes 6–10 contain 5 mM MgCl₂. Histone-bound and free DNA are indicated.

**Fig. 4.** Effect of MgCl₂ concentration, temperature, and KCl concentration on the disruption of nucleosomes. A, MgCl₂ concentration was varied from 0 mM (lanes 1–5) to 5 mM (lanes 6–10) to 12 mM (lanes 11–15) with KCN concentration and temperature held constant as described under “Materials and Methods.” B, temperature of incubation was changed from 0°C (lanes 1–5) to 25°C (lanes 6–10) to 37°C (lanes 11–15) with MgCl₂ and KCN concentrations held constant. C, KCN concentration was varied between 70 and 280 mM as indicated. MgCl₂ concentration was held at 5 mM, and the temperature was 30°C. Histone-bound and free DNA are indicated. Bar indicates the population of nucleosome cores to which a second histone octamer is bound (Ausio et al., 1984a, 1984b).
Fig. 5. Disruption is not dependent on DNA sequence or specific to monomeric core particles. Nucleosomes reconstituted onto the indicated DNA fragments and incubated under standard conditions (see "Materials and Methods"). Lanes 1–5 contain nucleosome core particles, and lanes 6–10 contain two core particles reconstituted onto a longer fragment of DNA. Histone-bound and free DNA are indicated.

Table II
Relative levels of nucleosome disruption

| Sample                  | % Disruption (normalized) |
|-------------------------|---------------------------|
| Intact octamers         | 71                        |
| Tail-less octamers      | 0                         |
| 0 mg MgcI₂             | 0                         |
| 3 mg MgcI₂             | 0                         |
| 5 mg MgcI₂             | 1.5                       |
| Intact octamers:        |                           |
| 0 mg MgcI₂             | 0.4                       |
| 5 mg MgcI₂             | 76                        |
| 12 mg MgcI₂            | 88                        |
| 0°C                    | 55                        |
| 25°C                   | 44                        |
| 37°C                   | 63                        |
| 70 mg KCl              | 36                        |
| 140 mg KCl             | 84                        |
| 280 mg KCl             | 92                        |
| TRJ₄A-monomer          | 74                        |
| 5 S RNA-dimer          | 95                        |

An increase in divalent or monovalent cation concentrations will significantly influence the stability of histone-DNA interactions at dilutions of nucleosomes commonly used in transcription factor binding experiments.

Our next experiments examined the role of temperature and KCl concentration in nucleosome dissociation. In agreement with earlier work (Ausio et al., 1984b) we find that an increase in temperature to 37 °C and monovalent cation concentration (KCl) to 280 mM further destabilizes nucleosome cores (Fig. 4, B and C). An increase in divalent or monovalent cation concentrations will contribute to the release of the core histone tails from stable interaction with nucleosomal DNA (Walker, 1984). This release might facilitate nucleosome disruption, potentially by allowing the tails to make contacts outside of the nucleosome. In these studies we have also found an example of two octamers bound to a single DNA fragment (Fig. 4C, lanes 1, 5, and 9) as previously reported (Ausio et al., 1984a, 1984b). The upper complex is selectively destabilized by dilution, reflecting the weaker association of the second histone-octamer with DNA (Ausio et al., 1984b).

Finally we made use of two additional specific chromatin substrates to show that a mononucleosome containing sequences from the Xenopus TRJA promoter (Ranjan et al., 1994) (Fig. 5, lanes 1–5) and a dinucleosome containing two reiterated 5S rRNA genes (Ura et al., 1995) are also destabilized by dilution (Fig. 5, lanes 6–10). These results are indicative of the generality of this nucleosome disruption phenomenon (Stacks and Schumaker, 1979; Cotton and Hamkal, 1981; Ausio et al., 1984a, 1984b).

Conclusions—We describe experiments that make use of standard conditions for examining the binding of trans-acting factors to DNA. Due to the sensitivity of the available gel shift and DNase I footprinting assays, together with inherent limitations in the availability of specific trans-acting factors and potential chromatin remodeling complexes, these assays typically utilize DNA at very low concentrations. We demonstrate here that the concomitant dilution of nucleosomes leads to loss of histone-DNA contacts under conditions that retain the binding of trans-acting factors.

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