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Activated RSC–Nucleosome Complex and Persistently Altered Form of the Nucleosome

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Summary

RSC, an abundant, essential chromatin-remodeling complex, related to SWI/SNF complex, binds nucleosomes and naked DNA with comparable affinities, as shown by gel shift analysis. The RSC–nucleosome complex is converted in the presence of ATP to a slower migrating form. This activated complex exhibits greatly increased susceptibility to endo- and exonucleases but retains a full complement of histones. Activation persists in the absence of ATP, and on removal of RSC, the nucleosome is released in an altered form, with a diminished electrophoretic mobility, greater sedimentation rate, and marked instability at elevated ionic strength. The reaction is reversible in the presence of RSC and ATP, with conversion of the altered form back to the nucleosome.

Introduction

Histones serve as general gene repressors, blocking the initiation of transcription in vitro (Knezetic and Luse, 1986; Lorch et al., 1987, 1988) and in vivo (Han and Grunstein, 1988). Repression by histones is opposed at some yeast promoters by SWI/SNF complex (Winston and Carlson, 1992), and histone–DNA interactions in isolated nucleosomes can be perturbed by the purified complex (Cote et al., 1994; Tsukiyama et al., 1994; Owen-Hughes et al., 1996; Varga-Wiesz et al., 1997). Homologs of four SWI/SNF proteins have been identified in yeast and have been shown to form part of another multiprotein complex, termed RSC (Cairns et al., 1996). Like SWI/SNF, purified RSC perturbs the structure of nucleosomes; in contrast with SWI/SNF, which is scarce and nonessential, RSC is abundant and required for yeast cell growth. Homologs of SWI/SNF proteins occur in higher cells, and one *Drosophila* homolog complex, as well as two families of human homolog complexes (Imbalzano et al., 1994; Dingwall et al., 1995; Wang et al., 1996), has been described.

Perturbation of nucleosome structure by SWI/SNF-related complexes is revealed by increased accessibility to DNase I and to sequence-specific DNA binding proteins. Such "chromatin-remodeling" requires ATP hydrolysis, attributable to the Swi2/Snf2 component of SWI/SNF complex and to its homologs Sth1 in RSC, brahma in the *Drosophila* complex, and hBrg and hbrm in the human complexes. Another family of chromatin-remodeling complexes, isolated from *Drosophila* embryo extracts, is distinguished by the presence of a more distant homolog of Swi2/Snf2, termed ISWI. The first member of this family to be identified, NURF, also perturbs nucleosome structure, as shown in various ways, including increased accessibility to DNase I.

The mechanisms of these yeast "chromatin remodeling" complexes and of other such complexes isolated from higher cells (Tsukiyama and Wu, 1995; Ito et al., 1997; Varga-Wiesz et al., 1997) are unknown; no reaction intermediates or other dissection of the remodeling process have been described. Here we demonstrate the formation of a discrete RSC–nucleosome complex, its conversion by ATP hydrolysis to an activated state, and the subsequent release of an altered form of the nucleosome.

Results

RSC–DNA and RSC–Nucleosome Complexes

Binding of RSC to DNA was detected by gel electrophoretic mobility shift analysis with a 172–base pair fragment of the *Xenopus laevis* 5S rRNA gene (Figure 1, lane 1). This DNA fragment forms a well-positioned nucleosome, used previously for studies of SWI/SNF interaction (Cote et al., 1994). The association of most, if not all, RSC subunits was shown by staining the gel with Coomassie blue, which revealed a single band of protein at the same position as that of the RSC–DNA complex (data not shown). A requirement for stoichiometric RSC and DNA was consistent with a 1:1 ratio in the complex. An affinity of about 10^{-8} M^{-1} was estimated by quantitation of the gels.

Binding of RSC to a nucleosome assembled on the 172–base pair 5S rDNA fragment was also revealed by gel electrophoretic mobility shift analysis (Figure 1, lane 3). The mobilities of the RSC–DNA and RSC–nucleosome complexes were virtually identical, presumably because the electrophoretic properties were dominated by RSC, five times the size of the nucleosome. Indeed, the mobility of RSC alone was the the same (shown by silver staining the RSC complex run alone in an identical gel). Like the RSC–DNA complex, the RSC–nucleosome complex appeared to be 1:1, with an affinity of about 10^{-8} M^{-1}.

It was previously reported that SWI/SNF complex binds preferentially to four-way-junction DNA, leading to the suggestion that the complex might similarly interact with the hypothetical crossover point of DNA entering and exiting the nucleosome (Quinn et al., 1996). We therefore investigated the binding of RSC to a nucleosome lacking a crossover point. The 172–base pair 5S rDNA fragment used in this work was reduced to 147 base pairs, centered on the expected dyad of the nucleosome, to form a typical core particle containing only one and three-quarters superhelical turns and therefore no possible crossover of the DNA. RSC bound to this 147–base pair core nucleosome with an affinity

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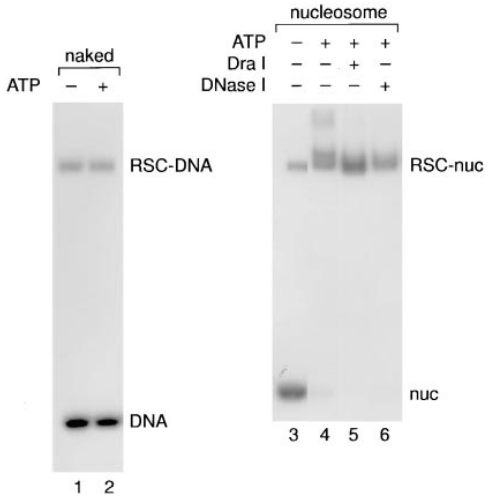

Figure 1. Formation of an Activated RSC–Nucleosome Complex

Nucleosomes (18 ng, lanes 3–6) assembled on the 172–base pair fragment labeled by filling in, or the naked DNA fragment from which they were derived (lanes 1 and 2), were combined with RSC (110 ng), with (+) or without (–) the addition of ATP, kept for 20 min at 30°C, and treated with DraI (35 U) for 10 min at 30°C or with DNase I (3 ng) as indicated for 40 s at room temperature, followed by gel electrophoresis. Singlet and doublet bands due to RSC–nucleosome complexes (RSC-nuc) and singlet bands due to RSC–DNA complex (RSC-DNA), to free nucleosomes (nuc), and to naked DNA (DNA) are indicated on the right.

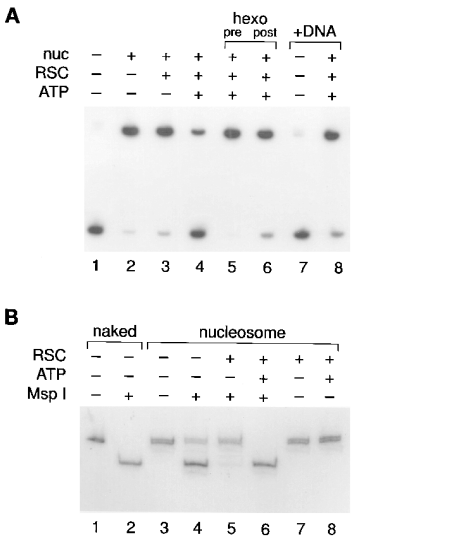

Figure 2. Restriction Endonuclease Digestion of an Activated RSC–Nucleosome Complex

(A) Effects of RSC and ATP on DraI digestion of nucleosomal DNA. Nucleosomes (as in Figure 1, 6 ng, except labeled with polynucleotide kinase and γ-^{32}P-ATP) or the naked DNA fragment from which they were derived (nuc +/–) was combined with 15 ng of RSC (+) or not (–) and ATP (+) or not (–), followed by incubation for 15 min at 30°C. DraI (60 U) was added, followed by incubation for 40 min, extraction of DNA, and electrophoresis in a 7% polyacrylamide gel. Hexokinase (hexo, 4 μg) and glucose (30 mM in reaction mixture) were added before (pre, lane 5) or after (post, lane 6) RSC, followed by incubation for 5 min at 30°C for the degradation of ATP before proceeding. Addition of hexokinase and glucose before RSC, which abolished its effect, was a control to demonstrate the complete degradation of ATP. Competing DNA (DNA, 2.5 μg of a closed circular plasmid) was added before DraI digestion (lanes 7 and 8) followed by incubation for 5 min at 30°C before proceeding.

(B) Effects of RSC and ATP on MspI digestion of nucleosomal DNA. Nucleosomes (as in Figure 1, 125 ng), or the naked DNA fragment from which they were derived, were combined in 25 μl of 10 mM Tris-Cl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT with 1 μg of RSC (+) or not (–), 0.5 mM ATP (+) or not (–), and 60 U of MspI (+) or not (–), followed by incubation for 1 hr at 37°, extraction of DNA, and electrophoresis in a 12% polyacrylamide gel. An autoradiograph of the gel is shown.

comparable to that of binding to the 172–base pair nucleosome (data not shown).

Activated RSC–Nucleosome Complex

Addition of ATP increased the affinity of RSC for the nucleosome but not for naked DNA (Figure 1, lanes 2 and 4) and converted a portion of the RSC–nucleosome complex to a more slowly migrating species, forming a doublet in the gel (Figure 1, lane 4; see also Figure 4, lanes 1 and 3). The doublet was not obtained with the RSC–DNA complex, nor with the use of a formaldehyde cross-linked nucleosome or a nonhydrolyzable analog of ATP (data not shown). Formation of the slower migrating or "activated" complex was due to RSC, and not to a contaminant in the RSC preparation, since activated complex-forming ability cochromatographed with RSC in the last step of purification and since activated complex formation was lost following immunodepletion with anti-Rsc11 antibodies (data not shown). The efficiency of conversion to the activated complex was at least as great for a 147–base pair core particle as for the 172–base pair nucleosome, showing again a lack of requirement for a crossover point in the nucleosomal DNA.

The slower migration of the activated complex in gels is indicative of a more extended conformation of the nucleosome, of RSC, or of both. Results of nuclease digestion point to unfolding of the nucleosome (but do not exclude a conformational change of RSC as well). The DNA fragment used in this work forms a well-positioned nucleosome, with cleavage sites for the restriction endonucleases DraI and MspI located about 15 and 41 base pairs from the dyad, respectively. Digestion with DraI destroyed the upper band of the RSC–nucleosome doublet but not the lower band (Figure 1, lane 5; see also Figure 4, lanes 2 and 4). Similar selectivity was found for digestion with DNase I, a classical marker of transcriptionally active chromatin: the upper band of the RSC–nucleosome doublet disappeared following DNase I digestion while the lower band was unaffected (Figure 1, lane 6).

Restriction endonuclease cutting was confirmed by extraction and analysis of nucleosomal DNA. DraI alone cleaved only 17% of the nucleosomal DNA during 40 min (Figure 2A, lanes 2 and 3; this cutting was probably due to a small amount of naked DNA contaminating the nucleosomal preparation), and no further cleavage was observed during more extended digestion (data not shown). By contrast, in the presence of RSC and ATP, DraI cut extensively, cleaving 86% of the nucleosomal DNA during 40 min (Figure 2A, lane 4), which again represented limit digestion. Similar results were obtained with MspI. In this case, 60%–70% of the nucleosomal DNA

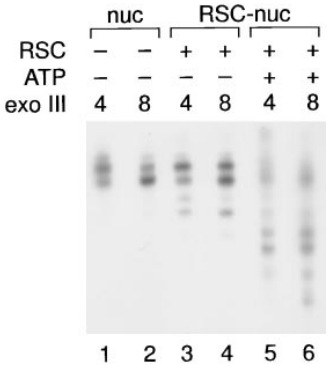

Figure 3. Exonuclease Digestion of Activated RSC–Nucleosome Complex

Nucleosomes were combined with RSC as in Figure 2A, with (+) or without (−) ATP as indicated, kept for 20 min at 30°C, and treated with exonuclease III (6 U) for 4 or 8 min as indicated, followed by the addition of EDTA to 5 mM and gel electrophoresis. DNA extracted from the singlet band containing RSC–nucleosome complexes from the reaction without ATP (RSC-nuc, lanes 3 and 4), the doublet band containing RSC–nucleosome complexes from the reaction with ATP (RSC-nuc, lanes 5 and 6), and the singlet band containing free nucleosomes (nuc, lanes 1 and 2) was analyzed in a 5% polyacrylamide gel containing 7 M urea.

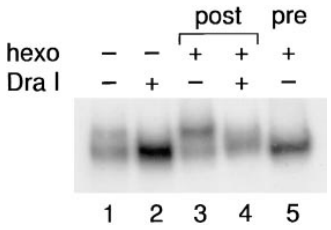

Figure 4. Persistence of Activated RSC–Nucleosome Complex in the Absence of ATP, as Revealed by Electrophoretic Mobility Shift and DraI Digestion

Nucleosomes (as in Figure 2A, 6 ng) were combined with RSC (15 ng) and ATP as in Figure 1 and treated where indicated with hexokinase and glucose for the removal of ATP as in Figure 2A. Other procedures were as in Figure 1. Only the upper region of the gel is shown, containing the bands due to RSC–nucleosome complexes.

was cut by the restriction enzyme alone (Figure 2B, lane 4), presumably reflecting the location of the cleavage site only 22 base pairs from an end of the nucleosome; exposure of the cleavage site may be attributed to thermal "breathing" or dissociation of the end from the histone octamer surface within the nucleosome. RSC inhibited this cutting completely (Figure 2B, lane 5), suggestive of RSC interaction near the end (in keeping with the dependence on a double-stranded character of the end noted below). Addition of ATP reversed the inhibition of MspI cutting by RSC (Figure 2B, lane 6), enabling cleavage of all the nucleosomal DNA.

RSC action also renders nucleosomes susceptible to exonuclease digestion. The boundaries of the nucleosome core particle are ordinarily impediments to digestion by exonuclease III, which trims the ends of the DNA rapidly to the 146–base pair core position and then degrades further much more slowly, with pauses every ten bases, reflecting the periodicity of DNA–histone contacts (Prunell and Kornberg, 1978). For studies of RSC–nucleosome complexes, reaction mixtures with or without ATP were treated with exonuclease III and resolved by gel electrophoresis. The RSC–nucleosome and residual free nucleosome bands were excised from the gel, and DNA was extracted and analyzed (the individual bands of the RSC–nucleosome doublet could not be cleanly separated and so were analyzed together). The RSC–nucleosome band from the reaction without ATP and the free nucleosome band exhibited the characteristic impediment to exonuclease III digestion (Figure 3, lanes 1–4), whereas the RSC–nucleosome doublet from the reaction with ATP showed much more extensive digestion (Figure 3, lanes 5 and 6). What little impediment remained in the case of the doublet may have been due to the lower (faster migrating) band of the doublet, presumed to contain an unaltered nucleosome.

The extensive alteration of nucleosome structure revealed by nuclease digestion raised the question of which, if any, histones remain associated with the activated complex. The answer could be obtained directly by extraction of proteins from the RSC–nucleosome band and analysis of proteins by SDS-PAGE. No difference in histone content of the band formed in the presence or absence of ATP was detected (data not shown).

Persistence of the Activated Complex and Release of an Altered Form of the Nucleosome

The structural perturbation of the nucleosome in the activated complex persisted following the removal of ATP. The complex continued to exhibit a diminished electrophoretic mobility and was still destroyed by DraI (Figure 4). Persistent structural perturbation was also apparent from DNase I footprint analysis (data not shown; previously reported for human SWI/SNF as well [Imbalzano et al., 1996]). A quantitative difference was, however, noted in the DraI cutting of nucleosomes formed on DNA with a single-stranded end (labeled with polynucleotide kinase) rather than a blunt end (labeled by filling in the end). The single-strand ended nucleosomes were less efficiently converted to activated complex (data not shown). Most of these nucleosomes were cut by DraI in the presence of RSC and ATP, but very little cutting was observed following the removal of ATP (compare Figure 2B, lanes 4 and 6). The majority of the nucleosomes were nonetheless cleaved by Dra I in the presence of ATP, which may be explained by catalytic action of RSC, binding a nucleosome, enabling cleavage by DraI, releasing the products, and repeating the cycle. ATP is required to drive the cycle, since following its removal, only the small fraction of nucleosomes in the activated complex at steady state is subject to digestion.

RSC was removed from complexes with labeled DNA or from complexes with labeled nucleosomes by competition with unlabeled DNA. All labeled DNA, and in the absence of ATP, all nucleosomes, were recovered in free form, as judged from mobility in a gel (data not shown). In the presence of ATP, the nucleosomes were released in altered form. About half of the altered nucleosomes were in a band at an intermediate position between RSC–nucleosome complexes and free nucleosomes (Figure 5A, left lane, band indicated by arrow).

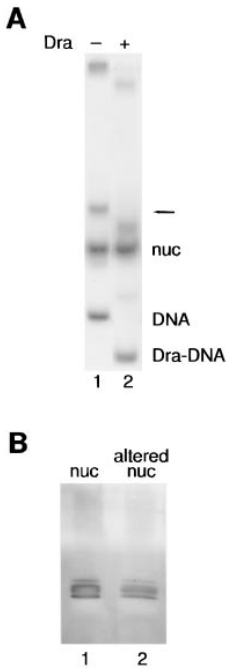

Figure 5. Persistently Altered Form of the Nucleosome

(A) Generation of the altered form by removal of RSC. Nucleosomes (6 ng) assembled on the 147–base pair fragment were combined with RSC (15 ng) and kept for 20 min at 30°C. DraI (40 U) was added (+) or not (−), followed by incubation for 40 min at 30°C, addition of closed circular plasmid (DNA, 3 μg), and incubation for a further 5 min at 30°C. Other procedures were as in Figure 1.

(B) Histone composition of altered nucleosomes. Reactions were as in (A) except that the levels of nucleosomes, RSC (omitted from lane 1), and plasmid DNA were five, six, and four times greater, respectively, and DraI digestion was omitted. Following electrophoresis as in Figure 1, proteins extracted from the gel bands were subjected to SDS-PAGE. Proteins were revealed by silver staining. The four bands visible in both lanes were coincident with those of a rat liver histone octamer marker (not shown). The intensities in lane 1 were somewhat greater than those in lane 2 because virtually all the material migrated in the nucleosome band extracted for lane 1, whereas only about one third of the material was in the intermediate band due to the altered nucleosome extracted for lane 2 (see Figure 5A, lane 1).

The remainder of the altered nucleosomes failed to enter the gel, due in all likelihood to aggregation, indicative of irreversible nucleosome unfolding. Additional bands due to free nucleosomes and DNA were caused by to incomplete conversion to the activated complex, as well as some free DNA present in the original nucleosome preparation. When the activated RSC–nucleosome complex was cleaved with DraI, followed by removal of RSC, both the intermediate band and the material at the top of the gel were shifted to faster migrating species (Figure 5A, right lane), showing that both were derived from the activated complex.

For identification of protein components, both the intermediate band and that due to the nucleosome were excised, extracted, and analyzed by SDS-PAGE. The intermediate band contained all four histones at approximately the same levels as the nucleosome (Figure 5B). No proteins other than histones were detected, but the presence of one or more RSC subunits in the intermediate band was not excluded.

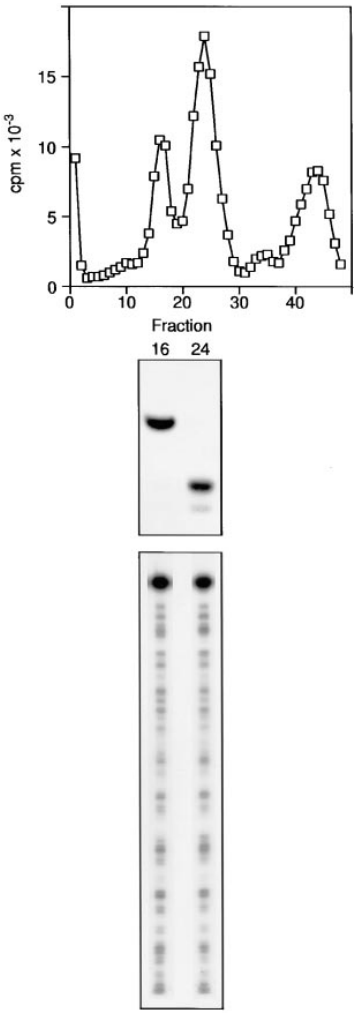

Figure 6. Maltose Gradient Sedimentation and DNase I Footprint Analyses of Altered Nucleosomes

A reaction mixture prepared and processed as in Figure 5A, lane 1, was sedimented in a maltose gradient. Gel electrophoresis and autoradiography as in Figure 1 identified the peak centered in fraction 16 with the intermediate band containing altered nucleosomes and the peak centered in fraction 24 with unaltered nucleosomes (middle panel). DNase I footprint analysis was performed on fractions 16 and 24 (lower panel).

To characterize further the altered nucleosomes in the intermediate band, they were isolated by sucrose gradient sedimentation (Figure 6). The order of sedimentation velocities was the inverse of the order of gel mobilities: sedimenting fastest, to near the bottom of the gradient, was the material that failed to enter the gel, followed by the altered nucleosomes, the unaltered nucleosomes, and finally, sedimenting slowest, free DNA, which ran fastest in the gel. Fractions containing altered and unaltered nucleosomes were subjected to DNase I footprint analysis (Figure 6). The footprints were almost the same and exhibited the enhanced cutting every ten bases characteristic of the DNase I digestion pattern of a nucleosome. The altered nucleosome thus differs from that in the activated complex, whose pattern of DNase I cutting more nearly resembles that of naked

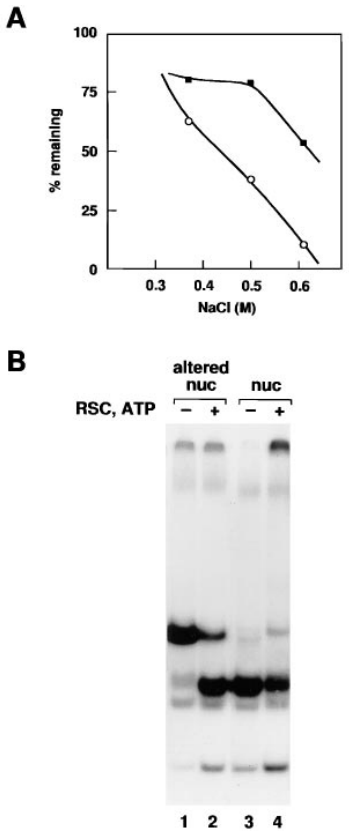

Figure 7. Characterization of the Altered Nucleosome

(A) Instability at elevated ionic strength. Reactions were performed as in Figure 5A, lane 1, followed by the addition of NaCl to the concentrations indicated, incubation for 5 min at 30°C, dilution with reaction buffer to 60 mM NaCl or less, and gel electrophoresis as in Figure 1. Radioactivity in bands due to the altered (open circles) and unaltered nucleosomes (closed squares) was quantitated with the use of a phosphorimager. Radioactivity lost from the nucleosomal bands appeared in the naked DNA band (not shown).

(B) Restoration of altered nucleosomes to the unaltered state by RSC and ATP. Maltose gradient fractions 16 and 24 from Figure 6, containing altered and unaltered nucleosomes, respectively, were treated with RSC and ATP (+) or not (−), followed by the addition of plasmid DNA competitor, as in Figure 5A, lane 1.

DNA (Cairns et al., 1996). Although the altered nucleosome has lost sensitivity to DNase I digestion, it nonetheless exhibits a diminished histone–DNA interaction, as shown by a loss of stability at elevated ionic strength. The altered nucleosome is almost completely destroyed by brief exposure to 0.5 M NaCl, whereas an unaltered nucleosome is unaffected (Figure 7A).

Although the structural change of the altered nucleosome is persistent, it can be reversed by readdition of RSC and ATP. RSC was removed by competition with unlabeled DNA following the reaction in order to reveal the nucleosomal products. The same pattern of products was obtained from the altered (Figure 7B, lane 2) and unaltered nucleosomes (Figure 7B, lane 4). In both cases, the predominant product was an unaltered nucleosome. The significance of this result is 2-fold: it provides further evidence that the altered nucleosome retains a full set of histones, and it rules out the possibility that the altered form represents an irrelevant, irreversible breakdown product of the nucleosome.

Discussion

RSC binds DNA and nucleosomes and disrupts histone–DNA interactions, as shown previously for other chromatin-remodeling complexes (Tsukiyama et al., 1994; Owen-Hughes et al., 1996; Quinn et al., 1996). Three further findings are reported here. First, a RSC–nucleosome complex is resolved as a discrete species by gel electrophoresis; previous attempts with other chromatin-remodeling complexes yielded only apparent aggregates, which failed to enter the gel. Second, an activated intermediate in the pathway of nucleosome disruption by RSC is detected, by a combination of gel electrophoresis and restriction enzyme digestion. Finally, nucleosomes are released from the activated complex in a persistently altered form, as shown by gel shift and sedimentation analysis.

Taken together, our findings reveal a cycle of nucleosome perturbation by RSC: treatment of the nucleosome with RSC and ATP leads to an activated state; removal of RSC results in an altered form; treatment again with RSC and ATP, followed by removal of RSC, restores the original nucleosomal configuration. Three states of the nucleosome are thus defined: activated, altered, and unaltered. Interconversion among these states by RSC and ATP is indicative of their physiologic relevance.

Our findings are informative about the chromatin remodeling process. The inhibition by RSC of MspI digestion near an end of a nucleosome is consistent with the idea of RSC invading the nucleosome from the ends. RSC may then create a bulge or wave by lifting the DNA off the histone surface, followed by propagation of the wave around the nucleosome in an ATP-dependent manner (Kornberg and Lorch, 1995; Pazin and Kadonaga, 1997). This mechanism of RSC action does not, however, readily explain such observations as the digestion of all activated RSC–nucleosome complexes by DraI in the absence of ATP and the persistence of an altered nucleosome following the removal of RSC. Further insight may be gained from the structure of the altered nucleosome. Although derived from the nucleosome in the activated complex, as shown by the results of DraI digestion, the altered form differs significantly from an activated nucleosome, for example, in sensitivity to cutting by DNase I. The altered form could be a conformational variant of the nucleosome, presumably more compact to explain its greater sedimentation velocity. Another possibility, accounting for both a greater sedimentation velocity and a diminished electrophoretic mobility, would be an increase in mass, due to association of additional protein(s) with the nucleosome. As mentioned above, association of one or more RSC subunits with the altered form was not detected but could not be ruled out. An increase in mass could also arise from dimerization of the nucleosome. For example, an end of DNA released from one nucleosome might bind to the histone surface exposed by the release of DNA from another. A transient effect of RSC on histone–DNA interaction would thus be trapped in a persistently altered state.

Experimental Procedures

Nucleosomes

Rat liver histone octamers were assembled as described (Lorch et al., 1987) on a 172–base pair AvaI–ScaI fragment of pIC5x207(E,X) (Cote et al., 1994), or on a 147–base pair AvaI–EcoRI fragment derived from pIC5x207(E,X) by PCR so as to preserve the location of the DraI site 15 base pairs from the dyad of the nucleosome. The 172–base pair fragment was labeled by filling in the AvaI end with α-^{32}P-TTP or by phosphorylation of the AvaI end with polynucleotide kinase and γ-^{32}P-ATP. The 147–base pair fragment was labeled by filling in the AvaI end with α-^{32}P-TTP. Maltose gradients (5%–30%), containing 25 mM HEPES (pH 7.6), 0.1 mg/ml BSA, 1 mM EDTA, 10 mM $NaHSO_3$, and proteinase inhibitors (Myers et al., 1997), were centrifuged in an SW41 rotor at 35,000 rpm for 16 hr at 4°C. Peak maltose gradient fractions of nucleosomes were used directly in reactions with RSC.

RSC

MonoS fractions of RSC, prepared exactly as described (Cairns et al., 1996), were used in all experiments. Similar results were obtained with multiple preparations, including RSC purified through an additional step of chromatography on TSK-heparin.

Activated RSC–Nucleosome Complex

Reaction mixtures (20 μl) contained 20 mM HEPES (pH 7.5), 0.5 mM ATP except where indicated, 4 mM $MgCl_2$, 30 mM potassium acetate, 75 μg/ml BSA. Nucleosomes and RSC were added in the amounts indicated. Electrophoresis was in 3.2% polyacrylamide gels containing 10 mM TrisCl (pH 7.5) and 1 mM EDTA, and autoradiographs of gels are shown.

Altered Nucleosomes

Reaction mixtures prepared as in Figure 5A, lane 1, except on ten times the scale, were sedimented in a maltose gradient as described above for the preparation of nucleosomes. Fractions of 0.25 ml were collected from the bottom of the gradient, and 20 μl of each fraction was counted. DNase I footprint analyses were performed as described (Cairns et al., 1996).

Recovery of DNA and Protein from Gels

Bands containing nucleosomes and RSC–nucleosome complexes were excised, minced, and extracted overnight with several volumes of 10% SDS, 0.25 M ammonium acetate, 10 mM TrisCl (pH 8), 1 mM EDTA. Extracted DNA was recovered by ethanol precipitation, dissolved in formamide, boiled, and analyzed in 7 M urea gels. Alternatively, bands were excised, minced, and extracted with 400 μl of 0.1% SDS, 75 mM NaCl, 50 mM TrisCl (pH 8.0), 0.1 mg/ml BSA, 0.1 mM EDTA overnight at 30°C. Gel particles were removed by centrifugation through a Costar SPIN-X 0.22 μm cellulose acetate filter unit, and proteins were precipitated with 4 vol of acetone for 2 hr at −80°C, washed with 80% acetone–20% extraction buffer, and dissolved in loading buffer for SDS-PAGE, performed as described (Lorch and Kornberg, 1994).

Acknowledgments

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Y. L. and R. D. K. dedicate this paper to the memory of Dr. Netanel Lorch, who with sword and pen wrote important pages in the history of Israel.

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