Chromatin Remodeling by SWI/SNF Results in Nucleosome Mobilization to Preferential Positions in the Rat Osteocalcin Gene Promoter*

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Changes in local chromatin structure accompany transcriptional activation of eukaryotic genes. In vivo these changes in chromatin organization can be catalyzed by ATP-dependent chromatin-remodeling complexes, such as SWI/SNF. These complexes alter the tight wrapping of DNA in the nucleosomes and can facilitate the mobilization of the histone octamer to adjacent DNA segments, leaving promoter regulatory elements exposed for transcription factor binding. To gain understanding of how the activity of SWI/SNF complexes may be modulated by the different DNA sequences within a natural promoter, we have reconstituted nucleosomes containing promoter segments of the transcriptionally active cell-type-specific osteocalcin (OC) gene and determined how they affect the directional movements of the nucleosomes. Our results indicate that SWI/SNF complexes induce octamer sliding to preferential positions in the OC promoter, leading to a nucleosomal organization that resembles that described in intact cells expressing the OC gene. Our studies demonstrate that the position of the histone octamer is primarily determined by sequences within the OC promoter that include or exclude nucleosomes. We propose that these sequences are critical components of the regulatory mechanisms that mediate expression of this tissue-specific gene.

Within the eukaryotic nucleus the DNA is organized as a highly structured nucleoprotein complex named chromatin. The nucleosome, the fundamental unit of chromatin, is formed by the association of a DNA segment of 147 bp around a histone octamer composed of two of each histone H2A, H2B, H3, and H4 (1, 2). The packaging of DNA sequences in nucleosomes and higher order chromatin structures has been implicated in the regulation of key events in eukaryotic cells such as replication and transcription (3, 4). The presence of nucleosomes is generally considered to block accessibility of most transcription factors to their cognate binding sequences. Moreover, gene activity is often accompanied by perturbations in the nucleosomal array, as evidenced by increased nuclease hypersensitivity of specific promoter and enhancer elements (3, 4). Multiple studies have established that there are intrinsic differences in the nucleosome binding capacity of different transcription factors. Although many factors cannot bind when their sites are assembled into nucleosomes, others can specifically recognize and interact with nucleosome-engaged binding sequences (3, 4), although with different degrees of affinity.

Studies based on nucleosome reconstitution assays in vitro have established the existence of DNA sequences that possess higher capacity to be organized as nucleosomal particles. The first segment reported to have this property was a region from the 5 S rRNA gene of sea urchin (5), which has become the standard for “nucleosome positioning” sequences. To date, no specific pattern can be associated with the ability to position nucleosomes. However, it appears that these sequences may exhibit certain parameters, such as the presence of AA duplets or AAA triplets, or CTG triplets every 10 or 11 bp (6, 7). Interestingly, there have also been reports suggesting the existence of “nucleosome-excluding” sequences, which include short repeats of adenine (A16) (8), long CCG triplet repeats (9), TGGA repeats (10), or the [(G/C)3NN]11 motif (11). These nucleosome-excluding sequences may lead to nucleosome-free promoter domains that could allow preferential access for transcription factors to their cognate elements.

During the last decade, a large family of protein complexes that function to promote transcription by altering chromatin structure have been described (3, 4, 12, 13). Among them is the SWI/SNF complex subfamily, which remodels chromatin in an ATP-dependent manner (3, 4, 12, 13). One feature of ATP-dependent chromatin remodelers is their ability to nucleosomize chromatin. Repositioning of histone octamers may occur along the same DNA segment by sliding (14–16) or to a nucleosome-free DNA segment by octamer transfer (17). The molecular mechanism by which the SWI/SNF complexes alter chromatin structure has not been definitively established; however, it has been proposed that they can bind to the linker region and push the DNA toward the nucleosome to slide the histone octamer (3, 12).

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Osteoblast-specific transcription of the rat osteocalcin (OC) gene is controlled by a series of modularly distributed basal and hormone-responsive promoter regulatory elements located within two DNase I-hypersensitive sites (−600 to −400 and −170 to −70) (18). The DNA segment between these two hypersensitive sites is organized as a nucleosome. The translational position of this nucleosome might reflect specific protein-DNA interactions occurring in the proximal promoter region that account for both formation of the proximal hypersensitive site and OC gene transcriptional activity (18–20). Alternatively, the specific location of this nucleosome might be the result of nucleosome-positioning sequences at this region of the OC promoter (21). To gain understanding about the basis for specific nucleosome positioning at the active OC gene promoter, we have characterized nucleosomes reconstituted using segments of this promoter. We find that SWI/SNF activity produces preferential histone octamer sliding to positions that resemble those found in intact osteoblast cells expressing the OC gene. This preferential octamer location is determined by specific sequences within the proximal OC promoter region that include or exclude nucleosomes.

EXPERIMENTAL PROCEDURES

**DNA Segments From the Rat OC Gene Promoter**—DNA fragments from the rat OC gene promoter containing the sequences −500 to −270, −500 to −87, −500 to −355, −451 to −306, −441 to −206, −380 to −145, −351 to −206, −351 to −116, −287 to −57, −257 to −106, −207 to −56, and −161 to −10 were generated by PCR using specific primers, one of which had been previously end-labeled with poly(ADP-ribose) polymerase (New England Biolabs, Beverly, MA) and [γ-32P]ATP (PerkinElmer Life Sciences), as previously described (21). To map nucleosome positions, labeled DNA fragments were obtained by PCR using specific primers and including [α-32P]dATP in the reaction mix as described before (21).

**Nucleosome Reconstitution**—Nucleosome reconstitutions were carried out by the histone octamer transfer method as previously described (21). Donor oligonucleosomes were obtained as described previously (21). To map nucleosome positions, 2 pmol of labeled DNA fragments and 6 μg of oligonucleosomes isolated from chicken red blood cells were used. In all the other reconstitution experiments, 1 pmol of acceptor DNA and 3 μg of oligonucleosomes were used. After reconstitution, the final composition of the nucleosome-containing buffer was 10 mM Tris-Cl (pH 7.4), 1 mM EDTA (pH 8.0), 5 mM DTT, 0.5 mM PMSF, 100 mM NaCl, 100 μg/ml bovine serum albumin, 10% glycerol, and 0.05% Nonidet P-40.

**Electrophoretic Analysis and Isolation of Nucleosome Populations**—Following reconstitution, the nucleosomes were analyzed by electrophoresis in non-denaturing 5% polyacrylamide (40:1 acrylamide:bisacrylamide ratio) gels and 0.5× TBE (45 mM Tris-Cl (pH 8.0), 45 mM Boric Acid, 1 mM EDTA), at 4 °C and 200 V. Gels were dried, and the nucleosome pattern as well as the efficiency of reconstitution were monitored using a Molecular Imager FX phosphorimaging device (Bio-Rad). Alternatively, the dried gels were exposed to Kodak X-OMAT films at −80 °C. The nucleosome populations obtained after the reconstitution were isolated and purified following protocols described by Studitsky et al. (22). Briefly, the different samples were concentrated up to 5-fold using centrificon-10 tubes (Amicon) and fractionated in non-denaturing polyacrylamide gels. Bands corresponding to naked DNA, lateral, and central nucleosome populations were cut from the wet gels and extracted with elution buffer (10 mM Tris-Cl (pH 7.4), 1 mM EDTA (pH 8.0), 5 mM DTT, 0.5 mM PMSF, 100 mM NaCl, and 100 μg/ml bovine serum albumin) by agitation overnight. Tubes were centrifuged 1 min at maximum speed in a microcentrifuge to decant gel pieces. The supernatant containing the nucleosomes was transferred to clean microtubes, and 0.25 volumes of elution buffer, including 50% glycerol and 0.25% Nonidet P-40, were immediately added. The samples were stored at 4 °C for up to 2 weeks until use.

**Mapping of Nucleosome Positions**—The purified nucleosome populations were subjected to micrococcal nuclease digestion for 5 min at 37 °C. The concentration of micrococcal nuclease used ranged from 0.1 to 1 unit/μl, depending on the particular nucleosome population and was determined by previous titration. The digested DNA products were purified and fractionated by electrophoresis in a 12% polyacrylamide gel (0.5× TBE) at 200 V. The 146-bp micrococcal nuclease-resistant band was detected by direct autoradiography, cut from the gel, and purified. The mapping of the nuclease-resistant fragment was then assessed by digestion with restriction enzymes. The enzymes chosen in each analysis varied according to the DNA segment and are explained in the corresponding Fig. legend. The digestion products were visualized by electrophoresis in a sequencing gel and autoradiography.

**Isolation of Nucleosome Remodeling Activity from Osteoblastic Cells**—A partially purified nucleosome remodeling complex analogous to hSWI/SNF and free of ISWI-containing complexes (see results shown in Fig. 5), was obtained according to a method described previously (23–25). Nuclear extracts from ROS 17/2.8 (rat osteosarcoma) cells were obtained as described (26), except that buffer C contained 0.6 m KCl instead of 0.42 m. Nuclear extracts were dialyzed against 20 mM HEPES (pH 7.9), 0.5 mM PMSF, and 0.5 mM β-mercaptoethanol, until KCl concentration reached 125 mM. Glycerol was added to the samples up to a final concentration of 20% (leaving KCl at a final concentration of 100 mM). The nuclear extract samples were then frozen in liquid nitrogen and kept at −80 °C until use. Prior to the first chromatographic step, nuclear extracts were centrifuged for 5 min at 5000 rpm. The supernatant was loaded on a phosphocellulose column and eluted with buffer A (20 mM HEPES (pH 7.9), 0.2 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 10 μg/ml TPCK, 10 μg/ml trypsin inhibitor, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and increasing concentrations of KCl (0.1, 0.3, 0.5, and 0.8 M). The protein concentration in each chromatographic fraction was determined by Bradford’s method, and the peak at 0.5 mM KCl was collected, dialyzed against buffer B (20 mM HEPES (pH 7.9), 0.1 mM KCl, 20 mM MgCl₂, 2 mM DTT, 0.1 mM EDTA, 0.5 mM PMSF, and 10% glycerol), and loaded onto a,

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*2 The abbreviations used are: OC, osteocalcin; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; nt, nucleotide(s); TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; ssDNA, single-stranded DNA; ROSF, partially purified SWI/SNF complex from ROS 17/2.8 cells.*
ssDNA column (Sigma). The sample was eluted using buffer B (also including a proteinase inhibitors mixture) with increasing concentrations of KCl (0.1, 0.3, and 0.5 M). The fractions were frozen in liquid nitrogen and stored at -80 °C. Enrichment in SWI/SNF complexes was monitored by analyzing aliquots from the different fractions by Western blot using antibodies against BRG-1 and by evaluating the presence of nucleosome remodeling activity.

Nucleosome Remodeling Reactions—The nucleosome remodeling assays were performed in 15 μl of remodeling buffer (60 mM KCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.5 mM PMSF, 2.5 mM DTT, 0.05% Nonidet P-40, 10% glycerol, 7 mM MgCl2, 4 mM ATP, and 100 μg/ml bovine serum albumin), including 0.73 μm of purified human SWI/SNF complex or 12.3 ng/μl (<&.6 μm SWI/SNF complex) of partially purified SWI/SNF complex from ROS 17/2.8 cells (ROSF), and 15.4 μm total nucleosome mixture (0.7 nm nucleosome probe, approximately). Samples were incubated for 30 min at 30 °C. Then, 750 ng of salmon sperm DNA (Sigma) and 500 ng of cold oligonucleosomes were added to the reaction mix and incubated for 30 min at 30 °C. The remodeled nucleosome samples were subsequently analyzed by electrophoresis in non-denaturant 5% polyacrylamide gels (0.5× TBE) or by direct restriction enzyme digestion (see below).

Restriction Endonuclease Enzyme Accessibility Analysis—Reconstituted nucleosomes or mock reconstituted DNA segments were digested with restriction enzymes for 20 min at 30 °C. The amount of each restriction enzyme used in the assays was determined previously as that sufficient to obtain a partial digestion of the naked DNA (close to 90%). Digestions were stopped by the addition of EDTA (20 mM final concentration). The nucleosome samples that had been previously subjected to remodeling reactions were incubated with 1 unit of aprotase (1 unit/μl) for 20 min at 30 °C, prior to the addition of restriction enzyme. Incubation with the restriction enzyme was carried out for 20 min at 30 °C, followed by the addition of 750 ng of salmon sperm DNA (Sigma), 500 ng of non-labeled oligonucleosomes and EDTA (20 mM final concentration). This reaction mix was incubated for 30 min at 30 °C. All samples were analyzed by electrophoresis in a 5% polyacrylamide gel as described above. The percentage of digestion was determined using a Molecular Imager FX (Bio-Rad).

Chromatin Immunoprecipitation Analysis—Chromatin immunoprecipitation studies were performed as described earlier (27) with modifications. All the steps were performed at 4 °C. ROS 17/2.8 cell cultures (100-mm-diameter plates) were washed with 10 ml of phosphate-buffered saline, scraped off in the same volume of phosphate-buffered saline, and collected by centrifugation at 1,000 × g for 5 min. The cell pellet was resuspended in 3 ml of lysis buffer (50 mM Heps (pH 7.8), 20 mM KCl, 3 mM MgCl2, 0.1% Nonidet P-40, and a mixture of proteinase inhibitors) and homogenized in a Dounce homogenizer using the loose pestle. The nuclear fraction was collected by centrifugation at 1,000 × g for 5 min and then incubated with the restriction endonuclease StuI (500 units/ml for 30 min at 37°C) according to previous reports (18). This enzyme cleaves between the proximal OC promoter region and the nucleosome positioned immediately upstream, therefore dividing the promoter and preventing that chromatin fragments containing sequences recognized by more than one specific set of primers are amplified. The reaction was stopped by the addition of 25 mM EDTA (final concentration), and the digested nuclear fraction was collected by centrifugation at 1,000 × g for 5 min and resuspended in 3 ml of sonication buffer (50 mM Heps (pH 7.9), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate acid, 0.1% SDS, and a mixture of proteinase inhibitors). To reduce the length of the chromatin fragments to ~300 bp or smaller (confirmed by electrophoretic analysis and PCR amplification (not shown), the extract was sonicated with a Misonix sonicator (model 3000), using ten 15-s pulses at 30% power. After centrifugation at 16,000 × g, the supernatant was collected, frozen in liquid nitrogen, and kept at -80 °C. An aliquot was used for A260 measurements. Extracts (6 units of A260) were resuspended in sonication buffer to a final volume of 500 μl. The samples were pre-cleared by incubation with 30 μl of protein A/G-agarose beads pre-blocked with bovine serum albumin (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min at 4 °C with agitation. After centrifugation at 1,000 × g for 5 min, the supernatant was collected and immunoprecipitated with either an anti-histone H3 polyclonal antibody (Santa Cruz Biotechnology, FL-136), anti-histone H4 polyclonal antibody (Santa Cruz Biotechnology, H-97), anti-acetylated histone H3 polyclonal antibody (Upstate Biotechnology, 06-599), or anti-acetylated histone H4 polyclonal antibody (Upstate Biotechnology, 06-866). As a control for nonspecific precipitation, normal rabbit-purified IgG fraction (Santa Cruz Biotechnology, sc-2027) was used. The immunocomplexes were recovered with the addition of 30 μl of protein A-agarose beads and subsequent incubation for 1 h at 4 °C with agitation. The complexes were washed twice with sonication buffer, twice with sonication buffer plus 500 mM NaCl, twice with LiCl buffer (100 mM Tris-HCl (pH 8.0), 500 mM LiCl, 0.1% Nonidet P-40, and 0.1% deoxycholic acid), and twice with dialysis buffer (2 mM EDTA and 50 mM Tris-HCl, pH 8.0), with the solution incubated at each washing for 5 min at 4 °C. The histone-DNA complexes were then eluted by incubation with 100 μl of elution buffer (50 mM NaHCO3 and 1% SDS) for 15 min at 65 °C. After centrifugation at 1,000 × g for 5 min, the supernatant was collected and incubated with 10 μg of RNase A per ml for 1 h at 42 °C. The proteins were then digested with 200 μg/ml proteinase K for 2 h at 50 °C. The DNA was recovered by phenol-chloroform extraction and ethanol precipitation using tRNA (5 μg/ml) as a precipitation carrier. The PCR conditions used to evaluate the OC proximal and distal promoter regions were reported previously (27). To amplify the precipitated OC promoter sequences the following primers were used: forward (−315) 5’-CGATTTTAGATCTCGTACCTCCTTAGG-3’; reverse (−206) 5’-GACTGGCCAGCAGCCCTTCT-3’ or reverse (−28) 5’-TATATCCACTGCCTGAGGC-3’; forward (−148) 5’-AGCTGAGTCAACACCAACC-3’; and reverse (−57) 5’-GCAGAGGGGCGAGGATGCT-3’.

RESULTS

The Proximal Region of the Rat Osteocalcin Gene Promoter Contains a Nucleosome-positioning Sequence—Multiple studies have established that following nucleosomal reconstitution in vitro, DNA segments larger than 150 bp (e.g. 230 bp) tend to
position the histone octamer at their ends (5'- or 3'-end) either immediately after reconstitution or after thermal redistribution, because these are the thermodynamically most stable conformations (28–30). This result is valid for any given DNA fragment unless it possesses a nucleotide sequence that favors a preferential position for the histone octamer different from the ends. We have previously reported that nucleosome reconstitution of segments from the proximal rat OC gene promoter results in a non-random positioning of the histone octamer (21). This finding suggests that there may be sequences within this region of the promoter that preferentially organize nucleosomes. To address this hypothesis we initially tested the -287/-57 OC proximal promoter segment, which encompasses all the basic tissue-specific regulatory elements (19, 20).

FIGURE 1. The DNA segment from the OC gene promoter spanning the sequence -287/-57 reconstitutes as two principal nucleosomal populations. A, a labeled DNA segment containing the sequence -287/-57 from the rat OC promoter was reconstituted as a mononucleosome by the histone octamer transfer method described under “Experimental Procedures.” The reconstituted samples were analyzed by electrophoresis in non-denaturing polyacrylamide gels (5%) and visualized in a phosphorimaging device. Slower and faster migrating nucleosomes were gel-isolated as described under “Experimental Procedures” and then re-analyzed by electrophoresis (lanes 3–5). Lane 1, mock reconstituted DNA -287/-57 segment; lane 2, -287/-57 segment assembled as a nucleosome; lane 3, gel-purified non-assembled -287/-57 DNA segment; lane 4, purified faster migrating nucleosome, and lane 5, purified slower migrating nucleosome. The predicted nucleosomal populations are shown at the right of the gel. B, the purified nucleosomal populations were mapped as described under “Experimental Procedures.” For the faster migration particle (lanes 3–6) cleavage products from incubation of the 146-bp micrococcal nuclease-resistant core with StuI are the major segments of 126 and 20 nt, and the minor segments of 105 and 41 nt (lane 4). Incubation with PstI leaves the 146-nt undigested fragment as the major product and the segments of 89/85 and 61/57 nt (cohesive end cleavage) as the minor products (lane 5). Similarly, cleavage with FspI leaves the 146-nt undigested fragment as the major product and the segments of 99 and 47 nt as the minor products (lane 6). For the slower migrating nucleoparticle (lanes 7–10), cleavage with StuI generates fragments of 126, 105, 89, and 57 nt (lane 8). Cleavage with PstI produces fragments of 146, 109/105, 89/85, and 61 nt (lanes 9). Similarly, cleavage with FspI produces an undigested 146-nt major segment and minor bands of 99 and 47 nt (lane 10). Lane 1 contains a mixture of the -287/-57 segment undigested or digested with StuI. Lane 2, contains a mixture of the -287/-57 segment digested with PstI or with FspI. Lanes 3 and 7, correspond to the undigested micrococcal nuclease-resistant fragment. Diagrams representing the principal positions adopted by the nucleosomes along the -287/-57 fragment are shown in C (for the faster migrating nucleosome) and D (for the slower migrating nucleosome).
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Fig. 1 shows that nucleosome reconstitution of this fragment generates two main bands as determined by native PAGE (Fig. 1A, lane 2). These bands reflect at least two principal histone octamer positions at either end of the −287/−57 segment (the faster migration band) and at the center of this segment (retarded migration band) (28, 31). Both nucleosome forms were isolated and purified as described under “Experimental Procedures” (Fig. 1A, lanes 4 and 5); thus indicating that they represent bona fide nucleosome particles that are stable at 4 °C for several days (data not shown).

The precise position of these nucleosomes along the −287/−57 DNA segment was determined (21). This approach involves digesting the isolated nucleosomes with micrococcal nuclease and mapping the position of the −146-bp micrococcal nuclease-resistant fragment by digesting with three different restriction enzymes. Mapping of the faster migrating nucleosome band confirmed that the histone octamer is positioned at both ends of the −287/−57 segment (Fig. 1B and C). However, the significant differences in intensity of the bands generated by cleavage with the restriction enzymes indicate that the principal nucleosomal location (which accounts for ~70% of the nucleosomal population) is at the 5′-end of the segment (Fig. 1B). For example, the bands of 126 nt and 20 nt corresponding to cleavage products generated after digestion of the micrococcal nuclease-resistant −287/−142 fragment with StuI are reproducibly and significantly more intense than the bands of 105 nt and 41 nt generated by cleavage of the micrococcal nuclease-resistant −202/−57 fragment with this same restriction endonuclease (Fig. 1B, lane 4). Mapping of the lower migration nucleosome band (Fig. 1B, lanes 7–10) confirmed that some of the histone octamers are located at the center of the −287/−57 segment, mainly positioned between −250 and −105 (Fig. 1D). Based on the restriction enzyme cleavage pattern (Fig. 1B, lanes 7–10) we determined that after isolation this central nucleosome population also exhibits a fraction of nucleosomes positioned at the ends of the −287/−57 segment (see also Fig. 1A, lane 5). This indicates that soon after purification (which proceeds at 4 °C), a fraction of the histone octamers are spontaneously mobilized toward the ends (28–30).

Our previous studies had established that in osteoblastic cells expressing OC, the promoter region of this gene exhibits a nucleosome positioned within the sequence −350 to −170 (18). We have further analyzed whether this segment of the OC promoter contains a specific sequence that contributes to the location of this nucleosome in vivo. We reconstituted as a nucleosome the segment −380/−145 (236 bp), which includes the region predicted to

FIGURE 2. The −380/−145 segment of the osteocalcin promoter positions a nucleosome in the center of the fragment. A, the labeled −380/−145 and −287/−57 segments were assembled as mononucleosomes and analyzed by non-denaturing PAGE. Lane 1, mock reconstituted −380/−145 segment; lane 2, −380/−145 segment assembled as a nucleosome; lane 3, mock reconstituted −287/−57 segment; and lane 4, −287/−57 segment assembled as a nucleosome. The predicted populations obtained after reconstitution, are shown at the right of the gel. B, the two major nucleosomal populations obtained after the reconstitution of the −380/−145 segment were gel-purified, concentrated, and analyzed by electrophoresis independently. Lane 1, mock reconstituted −380/−145 segment; lane 2, −380/−145 segment assembled as a nucleosome; lane 3, gel-purified non-assembled −380/−145 DNA segment; lane 4, faster migrating nucleosomal population; lane 5, slower migrating nucleosomal population. The predicted nucleosomal populations are shown at the right of the gel. The purified nucleosomal populations were mapped as shown in Fig. 1. Diagarms representing the principal positions adopted by the nucleosomes along the −380/−145 fragment are shown in C–E.
FIGURE 3. The osteocalcin promoter contains a nucleosome-positioning sequence. A, schematic representation of the OC gene promoter, showing the proximal (pDHS) and distal (dDHS) hypersensitive sites and the positioned nucleosome (N). The predicted nucleosomal populations obtained after reconstitution of the segments −380/−145, −441/−206, and −351/−116 are shown below. The positions of the restriction cleavage sites for PvuII and StuI within the segments −441/−206 and −351/−116, respectively, are also shown. B, labeled OC promoter segments −380/−145 (left panel), −441/−206 (center panel), and −351/−116 (right panel), were assembled and analyzed by electrophoresis in non-denaturing polyacrylamide gels. In each gel: lane 1, mock reconstituted fragment; lane 2, assembled nucleosome. The predicted nucleosomal populations are shown at the right of each gel. C, OC promoter segments −441/−206 (lanes 1–4) and −351/−116 (lanes 5–8) were assembled as mononucleosomes and the main position adopted by the histone octamer determined by restriction enzyme cleavage. Lane 1, mock reconstitution of the −441/−206 segment; lane 2, −441/−206 segment assembled as a nucleosome; lane 3, mock reconstituted −441/−206 segment digested with PvuII; lane 4, −441/−206 nucleosome cleaved with PvuII; lane 5, mock reconstitution of the −351/−116 segment; lane 6, −351/−116 segment assembled as a nucleosome; lane 7, mock reconstituted −351/−116 segment digested with StuI; and lane 8, −351/−116 nucleosome cleaved with StuI.

FIGURE 4. The nucleosome-positioning sequence is required to assemble nucleosomes in the center of 231-bp DNA fragments from the OC gene promoter. Using as a template an OC promoter version where the sequence −343/−161 has been deleted and specific primers, the region encompassed between −500 and −87 (231 bp) was amplified by PCR. This segment, which includes recognition sites for the restriction enzymes DraIII (−460) and PvuII (−148) (shown at the top), was then assembled as a nucleosome as described under “Experimental Procedures.” The position of the histone octamer along the DNA fragment was determined by restriction enzyme cleavage assays as described in Fig. 3. Lanes 1, 3, and 5, mock reconstitution of the −500/−87343/−161 fragment; lanes 2, 4, and 6, reconstituted nucleosomes; lanes 3 and 4, digestion with the enzyme PvuII; lanes 5 and 6, digestion with the enzyme DraIII. The predicted nucleosomal populations are shown at the right of the gel.

contain the nucleosome-positioning sequence. As shown in Fig. 2, after nucleosomal reconstitution the −380/−145 segment exhibits an electrophoretic pattern including two major bands, each representing different histone octamer positions (see below). The slower migrating band represents >90% of the reconstituted particles (quantified by phosphorimaging analyses) and migrates to a position characteristic of nucleosomes localized at the center of the DNA fragment (Fig. 2A, lane 2). The higher mobility nucleosome band migrated to a position corresponding to histone octamers associated with the ends of the DNA fragment (Fig. 2A, lane 2). This pattern is exactly the opposite of that obtained with the −287/−57 segment (Fig. 2A, compare lanes 2 and 4), indicating that the −380/−145 fragment spans a sequence that forms stable nucleosomal particles at the center. This conclusion was further confirmed by the isolation, purification, and sub-
ingly, the gel-purified central nucleosome population does not exhibit mobilization toward the DNA ends either immediately after purification (Fig. 2B, lane 5) or after a short-term storage at 4 °C (data not shown). This result is completely opposite that of the mobilization observed on the -287/-57 nucleosome and represents an exception to the general tendency of the histone octamers to relocate toward the DNA ends (30).

Because the -351/-206 sequence appears to contain the critical information for positioning, we evaluated two additional promoter segments that span this sequence, the segment -441/-206 that leaves the nucleosome-positioning sequence at the 3′-end and the -351/-116 segment, which leaves this sequence at the 5′-end (Fig. 3A). As shown in Fig. 3B, after reconstitution these two 236-bp DNA segments exhibited an electrophoretic pattern that is the opposite of that of the -380/-145 segment, showing nucleosomal particles principally organized at the ends of the fragments. This was confirmed by restriction endonuclease digestion analysis (Fig. 3C). The restriction enzyme PvuII, which readily cleaves the mock reconstituted -441/-206 fragment (Fig. 3C, lane 3) at a site located at -279 (Fig. 3A), is unable to digest this same fragment while assembled as a nucleosome (Fig. 3C, lane 4). This result indicates that the nucleosomes are mainly organized at the 3′-end of the -441/-206 segment, thereby blocking cleavage by PvuII. On the other hand, the enzyme StuI readily cleaves the -351/-116 segment at a site located at -162, whether this fragment has been mock reconstituted or assembled as a nucleosome (Fig. 3C, lanes 7 and 8, respectively). This result indicates that, after reconstitution, the nucleosomes are principally organized at the 5′-end of the -351/-116 segment, thus always leaving the StuI site available for cleavage.

The contribution of OC promoter sequences downstream of -350 to nucleosome positioning was also assessed by including in our studies an OC promoter version where the segment -343/-161 has been deleted. Using this promoter construct as a template and specific primers that recognize sequences at -351 to -206 (90% of the nucleosomes) and -337 to -192 (10% of the nucleosomes) (Fig. 2, C and D, respectively). Similarly, it was determined that the higher mobility nucleosomal band represents nucleosomes almost entirely positioned at the 3′-end of the -380/-145 segment (Fig. 2E). Interestingly, the gel-purified central nucleosome population does not

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FIGURE 6. ROSF contains ATP-dependent nucleosome-remodeling activity. A, the −287/−57 OC promoter segment was assembled as a nucleosome and incubated with ROSF (left panel) or purified hSWI/SNF (right panel) in the absence or presence of ATP as described under “Experimental Procedures.” Left and right panels: lane 1, mock reconstituted −287/−57 segment; lane 2, mock reconstituted −287/−57 segment incubated with ROSF (or hSWI/SNF) and ATP; lane 3, −287/−57 nucleosome incubated with ATP; lane 4, −287/−57 nucleosome incubated with ROSF (or hSWI/SNF) and ATP. B, ROSF and hSWI/SNF remodel the −287/−57 in a dose-dependent manner. Reconstituted −287/−57 nucleosomes were incubated with increasing concentrations of either ROSF (left panel) or hSWI/SNF (right panel) in the presence or absence of ATP. All lanes contain −287/−57 nucleosome probe. Left panel: lane 1, nucleosome probe plus ROSF (2.46 ng/μl) plus ATP; lane 2, nucleosome probe plus ROSF (12.3 ng/μl) plus ATP; lane 3, nucleosome probe plus ROSF (37 ng/μl) plus ATP; lane 4, nucleosome probe plus ROSF (37 ng/μl) without ATP. Right panel: lane 1, nucleosome probe plus hSWI/SNF (0.37 ml) plus ATP; lane 2, nucleosome probe plus hSWI/SNF (0.57 ml) plus ATP; lane 3, nucleosome probe plus hSWI/SNF (1.47 ml) plus ATP; and lane 4, nucleosome probe plus hSWI/SNF (1.47 ml) without ATP.

-500 and −87, we amplified a 231-bp DNA segment, which was then assembled as a nucleosome (Fig. 4). It was found that deletion of the nucleosome-positioning sequence results in nucleosomes localized at the ends of the DNA fragment (Fig. 4, lane 2). Moreover, using restriction enzyme digestion analyses, it was determined that, in the absence of the −343/−161 sequence, the histone octamers are primarily associated with the 5'-end of the amplified segment. Thus, the enzyme PvuII readily cleaves the fragment at a site located at −148 (see Fig. 4, at the top), whether this segment has been mock reconstituted or assembled as a nucleosome (Fig. 4, lanes 3 and 4). On the other hand, the enzyme DraIII, which readily cleaves the mock-reconstituted fragment (Fig. 4, lane 5) at a site located at −460 (see Fig. 4, top) is unable to digest this same fragment while it is assembled as a nucleosome (Fig. 4, lane 6). Additionally, these results suggest that the sequence −160 to −87 has a reduced ability to assemble nucleosomes as compared with that exhibited by the sequence −500 to −343 (see results below).

Taken together, our results indicate that the OC promoter sequence −351/−206 preferentially positions histone octamers in vitro. Therefore, this sequence may be contributing significantly to the position of a nucleosome in this region of the OC promoter in osteoblastic cells expressing this gene.

SWI/SNF Activity Induces a Specific Octamer Sliding Along DNA Sequences From the Proximal Promoter Region of the Osteocalcin Gene—Members of the SWI/SNF family of chromatin-remodeling complexes alter chromatin structure by mobilizing nucleosomes in an ATP-dependent manner (3, 4, 12, 13). Although in recent years there has been a significant progress in understanding the molecular mechanism involved in SWI/SNF function, there is little information about the contribution of the nucleosome positioning or nucleosome-excluding sequences present in natural promoters to the SWI/SNF activity. Therefore, we used the proximal promoter region of the rat OC gene to evaluate whether the presence of the ISWI, the catalytic subunit of the ISWI family of chromatin-remodeling complexes (12). Instead, ISWI was found enriched in chromatographic fractions eluted from a P11 column using 0.8 M KCl (Fig. 5C, lower panel, lane 5). Interestingly, this fraction also contained BRG-1 (Fig. 5C, upper panel, lane 5) and other components of the SWI/SNF complexes (not shown), thus confirming previous reports indicating that mammalian cells contain populations of chromatin-remodeling complexes that elute with different chromatographic properties from P11 columns (23–25). Although the 0.8 M P11 fractions also contain strong nucleosome sliding activity (data not shown) they were not further considered in these studies. ROSF was also found depleted of the bone-related transcriptional regulators Runx2 and C/EBPβ (Fig. 5E). These two factors are key regulators of bone-specific OC gene transcription and have been shown to recruit chromatin-remodeling complexes such as SWI/SNF to bone-specific promoters (27, 33). Similarly, ROSF does not contain significant levels of β-actin (Fig. 5E) as has been shown for other purified SWI/SNF complexes (34, 35).

We next evaluated the chromatin-remodeling activity associated with ROSF by analyzing its ability to mobilize nucleosomes carrying sequences from the OC promoter. As shown in Fig. 6, incubation of the nucleosome containing the sequence −287/−57 with ROSF and ATP results in the conversion of the nucleosome to faster migrating species (−351/−206) together with other elements of this promoter influences SWI/SNF activity.

Following previously reported procedures (23, 24, 26, 32), we purified a nuclear fraction highly enriched in SWI/SNF activity from rat-derived osteoblastic cells (ROS 17/2.8) that actively express OC (Fig. 5A). As shown in Fig. 5B, chromatographic fractions eluted from an ssDNA column using 0.3 M KCl (fractions 1–3, Fig. 5B, lower panel) are enriched in BRG-1, the catalytic subunit of SWI/SNF. This fraction, designated ROSF, also includes other subunits of the SWI/SNF complex such as Brm, BAF-250, BAF180, BAF-170, BAF-155, BAF-57, and INI1 (Fig. 5D). ROSF did not contain detectable amounts of
one possibility is that these faster migrating species represent nucleosome mobilization by ROSF beyond the 5'-end. This nucleosome remodeling pattern is not unique for ROSF activity, because incubation of the −287/−57 nucleosome with purified human SWI/SNF complex (32) also results in a transition to faster migrating species (Fig. 6A, right panel, lane 4). Addition of increasing amounts of either ROSF or hSWI/SNF to the reaction mix further increases formation of the faster migration species (Fig. 6B, left and right panels, respectively). Thus, the nucleosome mobilization activity depends on the concentration of the remodeling complexes.

To confirm that these faster migration species were originated mainly by mobilization of the histone octamers beyond the 5'-end of the −287/−57 segment, we assessed changes in accessibility to the restriction enzyme Stul. As shown in Fig. 7A, Stul cleaves at −162, which is 20 bp within the nucleosome positioned at the 5'-end of the −287/−57 fragment. Mobilization of the histone octamer beyond the 5'-end would necessarily leave the Stul site more exposed to cleavage by this enzyme. To ensure that we were only evaluating the ability of Stul to cleave previously remodeled nucleosomes, it was necessary to prevent any further ROSF activity during the incubation with the restriction enzyme. This can be accomplished by incubating with apyrase, an enzyme that hydrolyzes the ATP present in the remodeling reaction and therefore inhibits SWI/SNF activity. As shown in Fig. 7B, Stul is unable to cleave the −287/−57 DNA segment assembled as a nucleosome (Fig. 7B, lane 5). This result confirms that the location of the Stul recognition site within the nucleosome reduces accessibility to the restriction enzyme. More interestingly, Stul reproducibly cleaves the faster migration species generated by ROSF activity (Fig. 7B, lanes 6 and 7), indicating that this band represents a mobilization of the nucleosome beyond the 5'-end of the −287/−57 OC promoter segment. Quantification of several independent experiments under the same experimental conditions and subsequent statistical analyses (Student's t test, Fig. 7C) indicated that incubation with ROSF also results in significant Stul cleavage of the band representing the lateral nucleosomal particle (Fig. 7B, compare lanes 6 and 7). This finding is in agreement with previous reports indicating that nucleosome remodeling mediated by SWI/SNF results in increased accessibility to restriction sites (39), especially when the sites are located proximal to the nucleosome edge. Nevertheless, there is a significantly higher effect of ROSF activity on the Stul digestion of the band representing the remodeled nucleosome (p < 0.01, Fig. 7C), further confirming that this band represents octamer mobilization toward the 5'-end.

Mobilization of Nucleosomes by SWI/SNF Activity Is Influenced by a Nucleosome-excluding Sequence Present in the Proximal Promoter Region of the Ostecalcin Gene—We next determined whether nucleosome mobilization by SWI/SNF beyond the 5'-end of the −287/−57 OC promoter segment requires the presence of a significant portion of the nucleosome-positioning sequence at that particular end. We assembled nucleosomal particles using different segments of this promoter, containing at one of their ends a part (−500/−270, see Fig. 8A) or the entire (−441/−206 and −351/−116, see Fig. 8B) nucleosome-positioning region. After reconstitution all three segments were assembled with most of the histone octamers bound to the ends of each fragment (Figs. 3 and 8). As shown in Fig. 8, nucleosome remodeling by ROSF resulted in octamer mobilization toward the center of the DNA segments (Fig. 8A, compare lanes 2 and 4), as the analyses were performed in par-
allel). Addition of increasing amounts of ROSF did not alter these mobilization patterns (not shown). Interestingly, the efficiency of ROSF to mobilize the histone octamers varies significantly among the different fragments. Thus, although the /H11002/500/ and /H11002/270 and /H11002/441/ and /H11002/206 nucleosomes are readily mobilized toward a central position (Fig. 8/ A, lane 2; Fig. 8/ B, lane 2), movement of the histone octamer along the /H11002/351/ and /H11002/116 segment occurs with a significantly reduced efficiency and results in octamers located at intermediate positions (Fig. 8/ B, lane 4, marked with arrows).

Our results demonstrate that the nucleosome-positioning sequence located between /H11002/351/ and /H11002/206 in the rat OC promoter is not sufficient to determine the final location of the histone octamer during nucleosome remodeling by SWI/SNF in vitro. Furthermore, these results suggest that sequences present within the /H11002/351/ and /H11002/116 segment, especially those located downstream of /H11002/206, partially block the mobilization of nucleosomes by ROSF. Interestingly, this region is also contained within the /H11002/287/ and /H11002/57 OC promoter segment, which exhibits a remodeling pattern that differs significantly from that of the other DNA segments tested.

It has been proposed that gene regulatory regions may contain nucleosome-excluding sequences that contribute to generate nucleosome-free domains and thus facilitating transcription factor binding and transcriptional regulation (8). We therefore analyzed whether ROSF-mediated nucleosome mobilization beyond the 5'-end of the /H11002/287/ and /H11002/57 segment and the weak mobilization observed on the /H11002/351/ and /H11002/116 segment, result from the presence of a nucleosome-excluding sequence immediately downstream of the nucleosome positioned at this 5'-end. We hypothesized that this type of sequence would decrease mobilization of the histone octamer by ROSF toward the center of the /H11002/287/ and /H11002/57 segment and therefore allow visualization of nucleosomes mobilized only in the opposite direction. To evaluate the presence of nucleosome-excluding sequences in this part of the OC promoter, we assembled nucleosomes using three partially overlapping DNA segments that together span the entire proximal region of the promoter.
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The Proximal Promoter Region of the Osteocalcin Gene Exhibits Reduced Association with Core Nucleosomal Histones in Osteoblastic Cells Expressing Osteocalcin—We have previously shown that in osteoblastic cells expressing OC, the proximal promoter region of the OC gene is remodeled, exhibiting a DNase I-hypersensitive site (−170 to −70) and absence of canonical nucleosomal organization (18, 19, 27). To confirm that this sequence of the OC promoter exhibits reduced association with the core histone proteins H3 and H4, we performed chromatin immunoprecipitation analysis in cells that are actively transcribing the OC gene. Nuclei isolated from confluent ROS 17/2.8 cell cultures were incubated with the restriction enzyme Stul (see “Experimental Procedures”), which can efficiently cleave its recognition site (−162) located in a nucleosome-free chromatin domain (see the top of Fig. 11A) (18). Subsequently, the chromatin was further fractionated down to small fragments (shorter than 300 bp) by sonication. As shown in Fig. 11A, precipitation with either anti-histone H3 or -histone H4 polyclonal antibodies, revealed that the OC gene promoter sequence located downstream from the positioned nucleosome (−148 to −57) exhibits a significantly decreased association with these nucleosomal histones (Fig. 11A, see lanes 3 and 4 in the first and second panels, and quantification in Fig. 11B). The required control using a nonspecific IgG fraction (Fig. 11, A and B) confirmed that these anti-histone H3 and H4 antibodies are recognizing and precipitating specific targets in the chromatin fraction. Similarly, the antibodies directed against acetylated histones H3 or H4 precipitate a significantly higher amount of chromatin fragments containing the sequence that spans the positioned nucleosome (−351 to −206), hence confirming that the region immediately downstream exhibits decreased interaction with nucleosomal histones (Fig. 11A, lanes 5 and 6 and quantification in Fig. 11B).

**DISCUSSION**

Transcription of the OC gene in terminally differentiated osteoblasts is controlled by a series of modularly distributed basal and hormone-responsive promoter elements located within two DNase I-hypersensitive sites (−600 to −400 and −170 to −70) (18). The DNA segment between these two hypersensitive sites is organized as a positioned nucleosome. We have suggested that the translational position of this nucleosome and the formation of the proximal hypersensitive site reflect,

(Fig. 9A). These segments contain 152 bp, and therefore allow the formation of one mononucleosome in a single position (21). Comparison of the efficiency at which each segment is reconstituted under equivalent experimental conditions was used as a direct indication of the presence of sequences with higher or reduced ability to assemble nucleosomes (6).

As shown in Fig. 9B, there is a marked difference in the extent to which each DNA segment can be reconstituted into nucleosomes. Although the −161/−10 segment reproducibly exhibits the highest degree of nucleosome assembly (69%, see quantification of several independent reconstitution experiments in Fig. 9C), the −207/−56 and −257/−106 segments show significantly lower reconstitution levels (33 and 27%, respectively, see Fig. 9C). These results indicate that the sequences contained within the OC promoter segment located downstream −206 and shortly upstream −106 possess a decreased ability to form nucleosomes in vitro.

Interestingly, sequences located upstream of the OC proximal promoter region (Fig. 10A) exhibit in most cases higher capacity to be assembled into nucleosomes. As shown in Fig. 10B, the segment −351/−206 spanning the nucleosome-positioning sequence shows the highest degree of reconstitution (96%) among all the DNA fragments analyzed. Similarly, the sequence between −500 and −355 exhibits high levels of reconstitution (86%).

![FIGURE 10. The distal OC gene promoter region assembles nucleosomes with high efficiency. A, diagram of the OC promoter region indicating the putative nucleosome positioning and excluding sequences. 146-bp fragments spanning distal OC promoter sequences used for the reconstitution experiments are shown below. B, OC promoter segments −500/−355 (left panel) and −351/−206 (right panel) were assembled as nucleosomes in parallel and analyzed as described under "Experimental Procedures." The reconstitution efficiency for each fragment was determined as indicated in Fig. 9. In all the gels lane 1, mock reconstitution and lane 2, assembled nucleosome.](image-url)
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**A**

![Diagram of the OC promoter region and nucleosome positioning](https://www.cgeb.trieste.it)

**B**

![Bar graph showing binding of histones H3 and H4](https://www.cgeb.trieste.it)

at least in part, protein-DNA and protein-protein interactions occurring within this region of the promoter (18–21).

Here, we have reconstituted into nucleosomes segments from the OC promoter and established that the region −351 to −206 contains a nucleosome-positioning sequence. Therefore, we propose that this sequence is a key component in the chromatin organization exhibited by the OC gene in transcriptionally active bone-derived cells. Comparative sequence analyses (data not shown) indicate that this nucleosome positioning region does not show significant homology to previously reported ones (5, 7). Similarly, this region of the rat OC promoter does not exhibit a specific pattern of di (AA, AG, or AT)- or tri (AAA)-nucleotides distributed every 10–11 bp, which have been associated with nucleosome-positioning sequences (6, 7). Together, these results indicate that in native promoters no unique DNA sequence pattern is associated with the ability to position nucleosomes and that this property may be related to other parameters. Moreover, Widom and co-workers (40) have established that standard sequence alignment methodologies do not apply for nucleosome-positioning sequences. Recently, the importance of DNA flexibility for nucleosomal structure was reported (1, 41). The results indicate that the higher the degree of flexibility shown by a given DNA fragment, the higher the probability that such segment forms stable nucleosomes. Interestingly, computer-based analyses (see www.cgeb.trieste.it (42, 43)) predict a higher degree of bendability or flexibility in the −351/−206 OC promoter segment with respect to the neighboring regions (data not shown). Therefore, we suggest that the ability of this region of the OC promoter to position a nucleosome both in vivo and in vitro may be related to this high flexibility.

We also report that the OC promoter region located downstream of the positioned nucleosome contains a nucleosome-excluding sequence. We have previously demonstrated that the proximal promoter region contains all the regulatory elements that are required for both basal transcription and formation of the proximal DNase I-hypersensitive site (−170 to −70) (19, 20). Among these elements are the sites recognized by the transcription factors Runx2 (−138 to −130) and C/EBPβ (−106 to −99), which have been shown to recruit chromatin-remodeling complexes such as p300-P/CAF and SWI/SNF to the OC promoter (27, 44). Therefore, we postulate that the presence of a sequence that poorly supports nucleosome formation contributes significantly to the formation of the proximal hypersensitive site by facilitating nucleosomal destabilization by the targeted chromatin-remodeling complexes.

This nucleosome-excluding sequence may also have a key role in the final position that the nucleosome adopts between the distal and proximal DNase I-hypersensitive sites in the OC promoter, because it provides a barrier for octamer sliding toward the excluding sequence. It has also been suggested that ATP-dependent nucleosome mobilization may be blocked by particularly rigid sequences that cannot be easily organized into nucleosomes (8, 14). We find that the nucleosome-excluding sequence present in the proximal OC promoter prevents octamer mobilization by SWI/SNF. Interestingly, we determined that SWI/SNF activity results in nucleosome mobilization in a direction that is opposite to this nucleosome-excluding region, leading to an octamer bound beyond the DNA end. Together, these results indicate that, within the cellular context, nucleosome sliding by ATP-dependent chromatin-remodeling complexes can be significantly influenced by the presence of nucleosome-positioning and nucleosome-excluding sequences. Therefore, analyzing the contribution of these sequences in eukaryotic promoters becomes a necessity to further understand chromatin remodeling in vivo.

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