Interaction of CBFα/AML/PEBP2α Transcription Factors with Nucleosomes Containing Promoter Sequences Requires Flexibility in the Translational Positioning of the Histone Octamer and Exposure of the CBFα Site†

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ABSTRACT: Chromatin remodeling at eukaryotic gene promoter sequences accompanies transcriptional activation. Both molecular events rely on specific protein−DNA interactions that occur within these promoter sequences. Binding of CBFα/AML/PEBP2α (core binding factor α/acute myelogenous leukemia/polyoma enhancer binding protein 2α) proteins is a key event in both tissue-specific and developmentally regulated osteocalcin (OC) promoter activity. To address linkage between chromatin organization and transcription factor binding, we reconstituted segments of the rat OC gene proximal promoter into mononucleosomes and studied binding of CBFα proteins. We analyzed binding of bacterially produced Cbfα2A and Cbfα2B, two splice variants of the human CBFα2 gene, and determined the effect of heterodimerization with the Cbf*β* subunit on binding activity. Our results indicate that binding of the truncated Cbfα2A protein to naked DNA is independent of Cbf*β* whereas Cbfα2A binding to nucleosomal DNA was enhanced by Cbf*β*. In contrast, the Cbfα2B interaction with either naked or nucleosomal DNA was strongly dependent on heterodimerization with the Cbf*β* subunit. Additionally, our results demonstrate that both Cbfα2A alone and Cbfα2B complexed with Cbf*β* can interact with nucleosomal DNA only if there is a degree of flexibility in the positioning of the histone octamer on the DNA fragment and exposure of the CBFα site. This situation was achieved with a DNA segment of 182 bp from the rat OC promoter that preferentially positions mononucleosomes upstream of the CBFα binding site and leaves this element partially exposed. Taken together, these results suggest that nucleosomal translational positioning is a major determinant of the binding of CBFα factors to nucleosomal DNA.

The interplay between transcription regulatory factors and chromatin regulates gene expression in eukaryotic cells. It has been traditionally assumed that the presence of nucleosomes blocks accessibility of transcription factors to their cognate binding sequences. Moreover, gene activation is often accompanied by modifications of the nucleosomal array, which can be detected as increased nuclease hypersensitivity of specific promoter and enhancer elements (*1*). The ability to address experimentally the molecular mechanisms by which chromatin remodeling occurs has led to the characterization of a large family of proteins and protein complexes that function to promote transcription by altering chromatin structure (*2*). The mechanism(s) by which these complexes function has (have) not been clarified. However, the resulting alterations in chromatin structure render DNA sequences containing regulatory elements accessible to transcription factors. It appears that the increase in accessibility may not involve removal of histones (*3*−*5*), suggesting that modifications in histone−DNA interactions produce a remodeled nucleosomal structure.

Several reports have suggested that the molecular mechanism of chromatin remodeling may not necessarily involve nucleosome disruption. Nucleosome sliding, i.e., the movement of nucleosomes along the DNA, can in principle occur in the absence of interacting proteins (*6*, *7*). Moreover, it has been recently reported that chromatin remodeling complexes such as NURF and CHRAC are able to reposition histone octamers to neighboring locations on the same DNA fragment, while retaining the full complement of histones (*8*, *9*). Thus, transcription factors may take advantage of these movements to integrate themselves into chromatin. Once integrated, these proteins function as boundaries that redirect nucleosome positions. Bradbury and colleagues (*10*) have reported that nucleosome movements along DNA can be restricted by the presence of histone H1. In addition, it is well established that in vivo histone H1 is significantly depleted from active genes (*11*).

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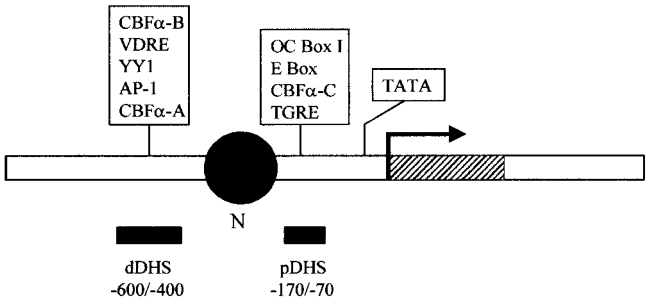

FIGURE 1: Schematic representation of the rat OC gene and flanking sequences. The hatched box represents the OC gene coding region (including exons and introns), and the horizontal arrow above the gene marks the direction of transcription. The filled circle represents a positioned nucleosome (N) and the filled boxes below, the distal (dDHS, −600 to −400) and proximal (pDHS, −170 to −70) DNase I hypersensitive sites. Key regulatory elements within the DNase I hypersensitive sites (e.g., CBFα site C, −138 to −130) are also indicated.

The rat osteocalcin (OC)[1] gene encodes a 10 kDa bone-specific protein that is induced in osteoblasts with the onset of mineralization at late stages of differentiation. Transcription of the OC gene is controlled by modularly organized basal and hormone-responsive elements (*12*), located within two DNase I hypersensitive sites that are present only in bone-derived cells expressing this gene (Figure 1; *13*, *14*). Thus, remodeling of the chromatin structure of the OC gene promoter accompanies the onset and increase in gene expression that occurs during osteoblast differentiation. It has been observed that the DNA segment located between the two DNase I hypersensitive sites in the OC gene is organized as a nucleosome (*14*). The translational positioning of this nucleosome may reflect protein−DNA interactions occurring in the proximal promoter of the OC gene, which account for the formation of the proximal DNase I hypersensitive site and for OC gene transcriptional activation (*15*). One principal tissue-specific regulatory element identified within the proximal region of the OC gene promoter is a recognition site for CBFα/AML/PEBP2α (core binding factor/acute myelogenous leukemia/polyoma enhancer binding protein 2α) (*16*−*20*).

CBFα/AML/PEBP2α factors function as heterodimers comprising two subunits in a 1:1 ratio; the α subunit contacts the DNA directly, and the *β* subunit forms a complex with the α subunit, but does not itself bind DNA (*21*, *22*). Association of the *β* subunit increases the DNA binding affinity of the α subunit (*23*, *24*). CBFα/AML/PEBP2α-related factors are expressed in tissues of the lymphoid, myeloid, and osteoblast lineages, where they are components of mechanisms mediating tissue-specific transcription (*21*, *22*). There are three genes designated CBFα1/AML-3, CBFα2/AML-1, and CBFα3/AML-2 which share a *runt* homology DNA binding domain, first observed in the *Drosophila runt* pair rule gene, and which encode highly conserved proteins (*21*). In addition, isoforms of the CBFα2/AML-1 protein are generated by alternative splicing (*22*). A shorter form of 250 amino acids (here called Cbfα2A) was the first to be described (*25*), contains the *runt* homology domain, and shows little or no ability to activate transcription (*26*−*28*). A longer form of 480 aa (here called Cbfα2B; *25*, *27*) contains a proline-, serine-, threonine-rich region (PST domain) that has been implicated in transcriptional activation (*26*). It has been suggested that Cbfα2A and Cbfα2B can act antagonistically for transactivation (*27*, *28*). Moreover, Cbfα2A was shown to bind in vitro to an Cbfα DNA binding site with significantly higher affinity than Cbfα2B. These results support the concept that Cbfα2A functions in vivo as a regulator of Cbfα2B-dependent transcriptional activation (*28*).

To understand transcriptional control of the OC gene within the intact nucleus, it is necessary to experimentally establish competency for interaction of CBFα transcription factors with cognate regulatory sites in a chromatin context. Therefore, we have reconstituted as mononucleosomes rat OC gene proximal promoter sequences containing a high-affinity CBFα binding site and evaluated the ability of purified Cbfα2A and Cbfα2B proteins to interact. We demonstrated that Cbfα2A binds to either naked or nucleosomal DNA independently of Cbf*β*, although interaction with nucleosomal DNA is enhanced by Cbf*β*. In contrast, Cbfα2B interaction with either naked or nucleosomal DNA is strongly dependent on the Cbf*β* subunit. Additionally, we report that interaction of both Cbfα2A and Cbfα2B/Cbf*β* with nucleosomal DNA requires a degree of flexibility in the translational position of the histone octamer and exposure of the CBFα binding site.

MATERIALS AND METHODS

Plasmids and Expression of Recombinant Proteins in E. coli. pGEX plasmids (Pharmacia Biotech, Uppsala, Sweden) containing the coding sequences of Cbfα2A (250 aa), Cbfα2B (480 aa), Cbf*β* (182 aa), Cbfα2$_{50-179}$ (*runt* homology domain, RHD), and Cbfα2$_{75-179}$ (DNA binding negative control) fused to glutathione-*S*-transferase (GST) were generously provided by Dr. Scott Hiebert (Vanderbilt University, Nashville, TN). The GST fusion proteins were obtained by transformation of the plasmids into *E. coli* BL21 and subsequent purification using a glutathione−Sepharose column (Pharmacia Biotech), according to the manufacturer's recommendations. GST-free Cbf*β* was obtained by cleaving the fusion protein attached to the column with 10 units of thrombin protease and subsequent elution. The purity and integrity of these proteins were evaluated by SDS−PAGE and Coomassie staining, and by western blot analysis using anti Cbfα polyclonal antibodies. The concentrations of the various proteins were determined by Bradford's assay and by a GST-specific colorimetric assay (Pharmacia Biotech). Aliquots of each protein were kept at −80 °C in the column elution buffer including 15% (v/v) glycerol and 100 mM KCl.

Isolation of Histone Octamer Donor Oligonucleosomes. Fresh blood was obtained from anesthetized chicken, mixed with anticoagulant solution [0.76% (w/v) sodium citrate, 1% (w/v) glucose], and kept at 4 °C until use. After centrifugation at 270*g* for 5 min, the pellet was resuspended and washed in RSB solution (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5

[1] Abbreviations: OC, osteocalcin; kDa, kilodalton(s); CBFα/AML/PEBP2α, core binding factor α/acute myelogenous leukemia/polyoma enhancer binding protein 2α; aa, amino acid(s); RHD, *runt* homology domain; GST, glutathione-*S*-transferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RSB, reticulocyte saline buffer; EMSA, electrophoretic mobility shift assay; TBE, Tris−borate−EDTA buffer; dDHS, distal DNase I hypersensitive site; pDHS, proximal DNase I hypersensitive site.

mM $MgCl_2$), and the cells were collected by centrifugation at 390*g* for 10 min each time. The pellet was then resuspended in 6 volumes of RSB supplemented with 0.5% (v/v) Nonidet NP-40 and 0.5 mM PMSF. The suspension was homogenized by vortexing gently, and then centrifuged at 750*g* for 10 min, and the pellet was washed 3 times with RSB−PMSF (0.5 mM). The nuclear pellet was subsequently resuspended in 8 volumes of digestion buffer (10 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM NaCl, and 0.5 mM PMSF) and quantitated by measuring the absorbance at 260 nm. Between 50 and 100 A_{260} units/mL of nuclei were incubated with 70 units/mL of micrococcal nuclease (Worthington, Freehold, NJ) for 15 min at 37 °C in digestion buffer. The reaction was stopped by the addition of EDTA and EGTA to final concentrations of 25 and 10 mM, respectively, and then centrifuged at 9600*g* for 20 min. To eliminate histones H1 and H5, the supernatant was dialyzed at 4 °C against dialysis buffer (0.7 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.5 mM PMSF, 0.5 mM β-mercaptoethanol) (*29*). After centrifugation of the dialysate at 150000*g* for 30 min, the supernatant was loaded and fractionated by chromatography in a Sepharose CL6B (Pharmacia) column (2.5 × 57 cm, 280 mL) as previously described (*30*). The column was preequilibrated and eluted with dialysis buffer. Fractions were collected, and the size of the DNA fragments was analyzed by electrophoresis in 1% (w/v) agarose gels. The presence, composition, and integrity of the core histones were determined by SDS−PAGE. Fractions containing oligonucleosomes with sizes ranging between 2 and 8 nucleosomes were pooled, dialyzed against dialysis buffer, but without NaCl, concentrated against sucrose (up to 1.0 μg of DNA/μL), dialyzed again, aliquoted, and kept at −80 °C until use.

Nucleosome Reconstitution. Labeled DNA fragments containing the sequences −287 to −106, −207 to −56, −257 to −106, and −257 to −57 from the rat osteocalcin (OC) gene promoter were generated by PCR using specific primers, one of which had been previously end-labeled with polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-^{32}P]ATP (New England Nuclear, Boston, MA). The plasmid pOC3.4 which contains 1.1 kb of the OC gene promoter was used as DNA template. The labeled fragments were purified by using G-50 Quick Spin Columns (Boehringer Mannheim) and then reconstituted into nucleosomes by the histone octamer transfer method or mock-reconstituted (negative control) as previously described (*30*). Successful nucleosome reconstitution was verified by analyzing samples by electrophoresis in native polyacrylamide gels and autoradiography and by evaluating resistance to digestion with micrococcal nuclease. In addition, the cleavage pattern generated by incubation of these mononucleosomes with DNase I and specific restriction enzymes was determined.

Protein−DNA Interaction Analysis. Binding of the bacterially expressed CBFα proteins to naked and reconstituted nucleosomes was carried out and analyzed by EMSA essentially as described earlier (*30*, *31*). The concentration used for each of the factors is indicated in the figure legends.

Footprinting Analysis. End-labeled mock-reconstituted DNA and reconstituted nucleosomes were incubated with increasing concentrations of DNase I for 2 min at room temperature (20 °C). Based on the observed digestion patterns, optimal results were obtained if incubations are performed in the presence of 0.01 (naked DNA) and 0.1 (nucleosomes) units of DNase I (not shown). The concentrations used for each transcription factor are indicated in the figures. The digested DNA samples were fractionated in 6% (w/v) sequencing gels and analyzed by autoradiography (*32*).

Restriction Endonuclease Accessibility Analysis. End-labeled mock-reconstituted DNA and reconstituted nucleosomes were incubated with the restriction enzymes (New England Biolabs, Beverly, MA) *Pst*I (2.5 units), *Stu*I (9 units), and *Fsp*I (5 units) in a 30 μL reaction volume, for 20 min at 30 °C. The buffer conditions used during these incubations were those provided by the supplier. The resulting DNA products were purified by phenol/chloroform extraction and ethanol precipitation. The various samples were then analyzed by electrophoresis in 8% (w/v) polyacrylamide gels at 4 °C with 1× TBE, and autoradiography. The cleavage efficiency was determined as described before (*31*).

Mapping of Nucleosome Positions. Mapping of the nucleosome positions on the reconstituted segments was carried out as previously described (*33*). Briefly, labeled DNA fragments were obtained by PCR using specific primers, the plasmid pOC3.4 as a template, and including in the reaction mix [α-^{32}P]dGTP. The labeled fragments were purified by using G-50 Quick Spin columns and then reconstituted into nucleosomes by the histone octamer transfer method or mock-reconstituted as previously described. The nucleosomes were then digested with micrococcal nuclease for 5 min at 37 °C. The digested DNA products were purified and fractionated by electrophoresis in polyacrylamide gels. The band corresponding to aproximately 150 bp was detected by autoradiography, cut from the gel, and purified. The mapping of the micrococcal nuclease-resistant fragment was then determined by digestion with one or two restriction enzymes, electrophoretic fractionation of the cleavage products in a sequencing gel, and autoradiography. After analysis of the restriction patterns in several trials, the most abundant nucleosome positions were determined.

RESULTS

CBFα/AML Proteins Do Not Bind to a CBFα/AML Site in a 152 bp DNA Fragment Assembled as a Mononucleosome. To gain insight into the molecular mechanisms that contribute to nucleosome remodeling at the OC gene promoter, we assembled as nucleosomes DNA segments containing key regulatory elements of the proximal promoter region of this gene. Subsequently, we analyzed specific protein−DNA interactions within this chromatin-like context. In the reconstitution experiments, histone H1- and H5-free oligonucleosomes isolated from chicken erythrocytes were used as a source of histone octamer donor (*29*, *30*). This protocol results in more efficient packaging of the DNA segments than reconstitution using purified histone octamers (data not shown).

Successful nucleosome reconstitution was verified by analyzing samples by electrophoresis in native polyacrylamide gels and autoradiography. As shown in Figure 2 (A−D), for all the segments analyzed in this study a high level of packaging (above 70%) was obtained. Additionally, efficient nucleosome reconstitution was assessed by evaluating resistance to digestion by micrococcal nuclease. As an

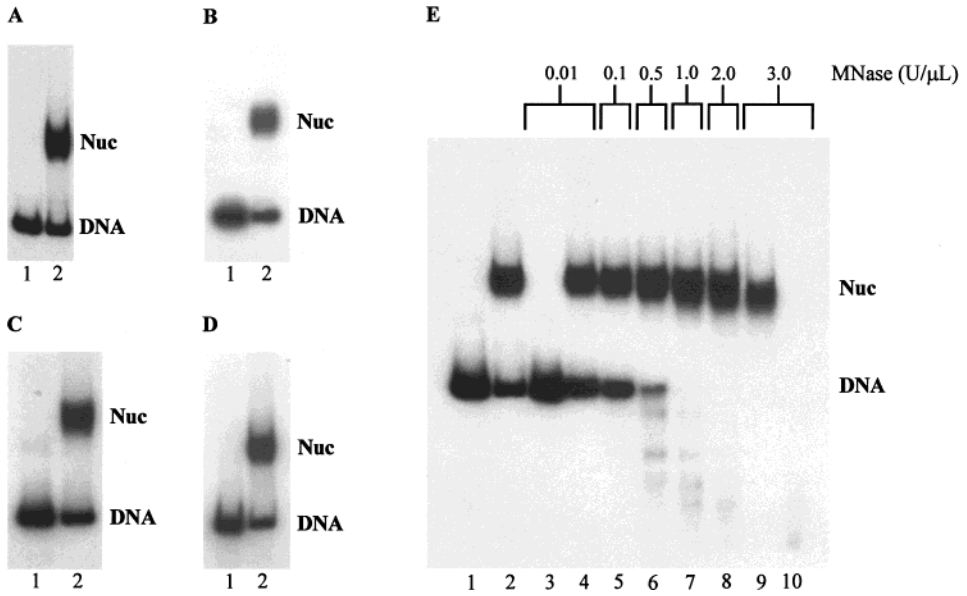

FIGURE 2: Reconstitution of nucleosomal particles. End-labeled DNA segments containing sequences −207/−56 (A), −257/−106 (B), −287/−106 (C), and −257/−57 (D) from the rat OC gene promoter were reconstituted as mononucleosomes by the histone octamer transfer method as described under Materials and Methods. The reconstituted samples were then analyzed by electrophoresis in nondenaturing polyacrylamide gels (5%) and visualized by autoradiography. In all four gels: lane 1 = mock-reconstituted DNA segment, lane 2 = DNA segment reconstituted as a nucleosome. (E) Successful nucleosomal reconstitution of the −287/−106 DNA segment evaluated by digestion with increasing concentrations of micrococcal nuclease at 37 °C for 5 min. The digestion products were subsequently fractionated in a nondenaturing polyacrylamide gel (5%) and visualized by autoradiography. Lanes 1, 3, and 10 = mock-reconstituted DNA segments; lanes 2, 4−9 = reconstituted nucleosomes. The concentration of micrococcal nuclease used in the reaction for each lane is indicated at the top. The position of the nucleosome (Nuc) and the free DNA (DNA) is marked on the left side.

example, Figure 2E shows that the −287/−106 fragment reconstituted as a nucleosome generated a 150 bp micrococcal nuclease-resistant segment when it was incubated with increasing concentrations of the enzyme. It should be noted that the free DNA fraction corresponding to the nonreconstituted 182 bp fragment is readily digested by a nuclease concentration 6-fold lower (Figure 2E, compare lanes 6 and 9). Similarly, mock-reconstituted −287/−106 fragment is completely digested at the enzyme concentration required to generate the 150 bp micrococcal nuclease-resistant particle (Figure 2E, compare lanes 9 and 10).

Full-length proteins Cbfα2A (250 aa), Cbfα2B (480 aa), and Cbfβ (182 aa) as well as polypeptides containing the Cbfα *runt* homology domain (Cbfα2_{50-179}) or residues 75−179 (Cbfα2_{75-179}, negative DNA binding control) (Figure 3A) were expressed in *E. coli* and subsequently purified as GST fusion proteins. The GST domain of Cbfβ was eliminated by cleaving with thrombin protease (see Materials and Methods). The DNA binding activity of these transcription factors as well as the capacity of Cbfβ to heterodimerize with the *runt* homology domain was evaluated by EMSA (electrophoretic mobility gel shift assay) and DNase I footprinting analysis (not shown). In addition, by testing GST-free Cbfα2A and Cbfα2B, it was determined that the presence of the GST domain fused to Cbfα2A and Cbfα2B affected neither their affinity for DNA nor their capacity to heterodimerize with Cbfβ (not shown).

The preparations containing the expressed proteins were analyzed by SDS−PAGE and Coomassie staining. As shown in Figure 3B, we obtained recombinant Cbfα2A, Cbfα2B, Cbfα2_{50-179}, Cbfα2_{75-179}, and Cbfβ preparations with reasonable purity. To determine that the low molecular weight band that coeluted with Cbfα2B was not a proteolytic product of this transcription factor but corresponds to free GST (lane 6), we performed Western blot analysis. As shown in Figure 3C, a specific anti-Cbfα polyclonal antibody (*17*) detected a single band corresponding to Cbfα2B (Figure 3C, lane 3). When an aliquot of the Cbfα2B sample was analyzed with an anti-GST antibody, we observed a lower band that comigrates with GST (Figure 3C, lanes 2 and 3). Western blot analysis using a specific antibody that recognizes the amino-terminal region of the Cbfα2A factor confirmed that the bacterially produced Cbfα2A contained a protein of the expected size (Figure 3D). Hence, the results indicate the presence of intact Cbfα2A, Cbfα2B, Cbfα2_{50-179}, Cbfα2_{75-179}, and Cbfβ factors in our recombinant preparations.

To investigate whether Cbfα2 proteins bind to nucleosomal DNA, we reconstituted a 152 bp fragment from the OC gene promoter (−207 to −56) which contains a Cbfα binding site (−138 to −130), shown to be a key regulatory element for the tissue-specific expression of this gene in bone cells (*16*, *18*). Consequently, this site was centered near the nucleosome dyad, at 75 bp from each of the ends (Figure 4A). Binding of GST-Cbfα2A and GST-Cbfα2B, alone or in the presence of equivalent concentrations of Cbfβ, to mock-reconstituted (naked DNA) and reconstituted nucleosomes was analyzed by DNase I footprinting.

As shown in Figure 4A, Cbfα2A was able to bind to naked DNA at low concentrations (75 nM), and this interaction was not dependent upon or enhanced by the presence of Cbfβ (compare lanes 2, 3, and 4). In contrast, Cbfα2B binding was significantly enhanced by the presence of Cbfβ (compare lanes 6, 7, and 8). Neither Cbfα2A nor Cbfα2B alone could bind to this fragment when it was reconstituted as a nucleosome (Figure 4A, lanes 10−17). Neither increasing the concentrations of these two transcription factors nor

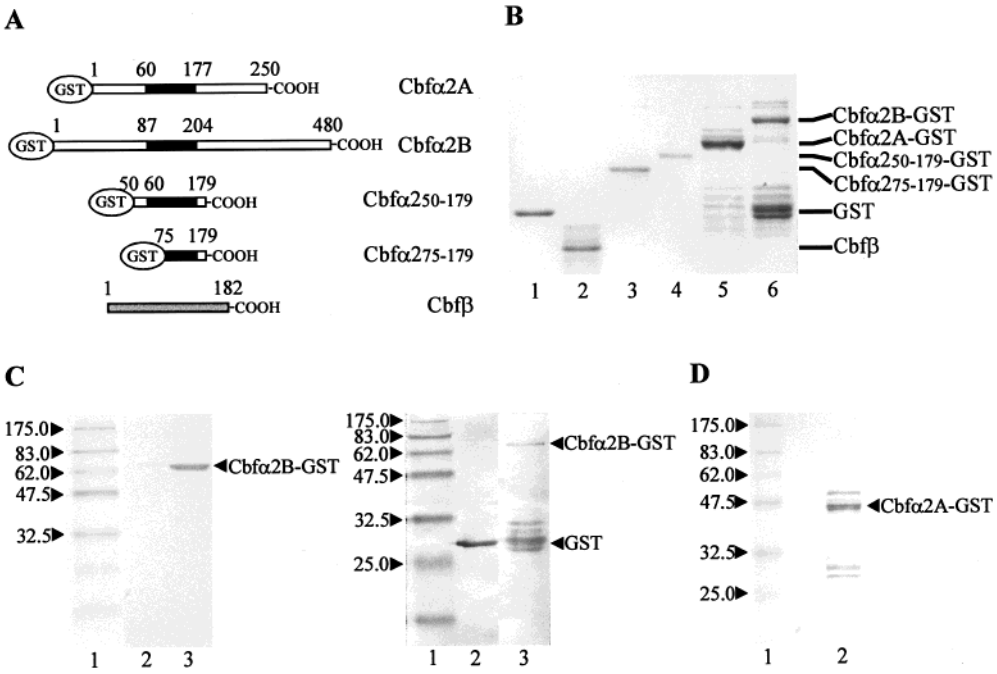

FIGURE 3: Expression of CBF proteins in *E. coli*. (A) Diagram of the GST-fusion proteins expressed in *E. coli*. For the Cbfα2 proteins, the filled rectangle represents the *runt* homology domain. The GST domain fused to Cbfβ was eliminated by cleaving with thrombin protease. (B) Coomassie-stained PAGE−SDS gel containing purified bacterially expressed CBF proteins. Lane 1 = GST, lane 2 = GST-free Cbfβ, lane 3 = GST-Cbfα2$_{75-179}$, lane 4 = GST-Cbfα2$_{50-179}$, lane 5 = GST-Cbfα2A, and lane 6 = GST-Cbfα2B. (C) Western blot analysis of the bacterially expressed GST-Cbfα2B protein. Left panel: Western blot revealed using an anti-CBFα polyclonal antibody, lane 2 = GST and lane 3 = GST-Cbfα2B; right pannel: Western blot revealed with an anti-GST polyclonal antibody, lane 2 = GST and lane 3 = GST-Cbfα2B. The position of the GST-Cbfα2B and GST proteins is marked on the right side of each blot. The sizes of the molecular weight bands are marked on the left. (D) Western blot analysis of the bacterially produced GST-Cbfα2A protein using an anti-Cbfα2 polyclonal antibody directed against the N-terminus of Cbfα2. The position of the GST-Cbfα2A protein is indicated on the right side. The sizes of the molecular weight bands are marked on the left.

adding Cbfβ promoted a significant change. Thus, the periodic cutting pattern every 10 bp, characteristic of nucleosomal DNA cleaved by DNase I, was not altered by the presence of Cbfα2A or Cbfα2B with or without Cbfβ, indicating the absence of significant interaction.

Successful nucleosome reconstitution as well as persistence of its structure following the addition of Cbfα factors was also confirmed by restriction endonuclease digestion analysis. It is well established that accessibility to restriction enzyme cleavage is markedly reduced when cognate recognition sequences are organized into nucleosomes (*34*). As shown in Figure 4B, cleavage by the enzymes *Pst*I and *Fsp*I was significantly reduced following nucleosomal reconstitution of the −207/−56 fragment. In addition, the cutting efficiency by these two enzymes was not significantly altered by the addition of Cbfα$_{50-179}$, Cbfα2A, or Cbfα2B/Cbfβ, thus confirming an absence of interaction.

It has been reported that the distance of a DNA element from the edge of a nucleosome becomes an important consideration when the binding of a transcription factor to nucleosomal DNA is evaluated (*30*, *31*). To test this possibility, we assembled the segment −257 to −106 from the OC gene promoter, locating the Cbfα site 20−24 bp from the edge of the nucleosome. For several transcription factors, this distance is sufficient to allow specific interaction (*30*, *31*). However, the analysis showed no significant interaction of either Cbfα2A or Cbfα2B with the nucleosomal DNA. Moreover, the presence of Cbfβ did not enable Cbfα2A or Cbfα2B to bind to the CBFα site when located close to the edge of the nucleosome (data not shown).

Taken together, these results confirm the high affinity of Cbfα2A for Cbfα binding sites previously reported (*28*). Our findings also indicate that Cbfα2A is capable of interacting strongly and specifically with this Cbfα site in the absence of Cbfβ, at least within the context of the rat OC gene promoter sequence. In addition, we observed that neither Cbfα2A nor Cbfα2B is able to bind to the proximal Cbfα site of the OC gene promoter when organized as a histone H1-free nucleosome.

Flexibility in the Histone Octamer Translational Position Allows Specific Binding of Cbfα2 Proteins to a Cbfα Site Reconstituted as a Nucleosome. It has been reported that in the absence of histone H1, nucleosomes can move or slide on DNA (*6*, *7*, *10*). Thus, transcription factors could take advantage of these movements to interact with their cognate binding sites, especially when these sequences are located proximal to the edge of the nucleosome.

We tested the possibility that spontaneous sliding or specific positioning of the histone octamer could facilitate binding of the Cbfα2 factors to nucleosomal DNA. We assembled a longer (182 bp) DNA fragment from the OC gene promoter (−287 to −106, see Figure 5A) as a nucleosome and determined whether this structure permitted accessibility of Cbfα2 proteins to the Cbfα binding site.

Previous studies have determined that when nucleosomes are reconstituted on linear DNA fragments longer than 150 bp, there is a greater preference for nucleosome location at the center or either end of the fragment (*35*). Accordingly, our rationale was that after reconstitution the Cbfα site would be located either about 24 bp upstream (nucleosome a, Figure

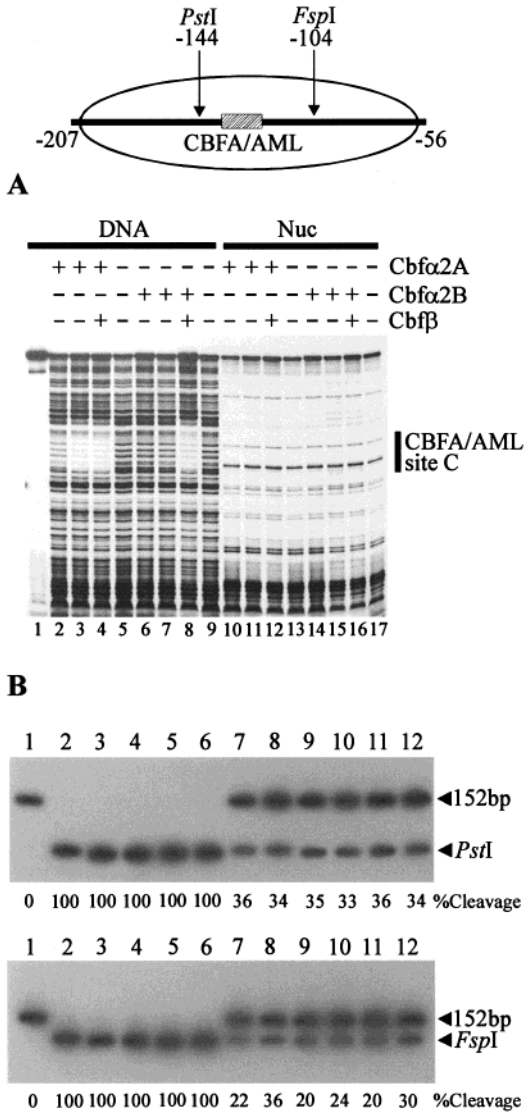

FIGURE 4: (A) DNase I footprinting analysis of GST-Cbfα2A and GST-Cbfα2B binding to the −207/−56 naked DNA fragment (lanes 1−9) or reconstituted as a nucleosome (lanes 10−17). A concentration of 75 nM of each transcription factor was used in lanes 2, 6, 10, and 14. Concentrations of 300 nM were used in the binding reactions shown in lanes 3, 4, 7, 8, 11, 12, 15, and 16. In lanes 2−9, the DNA samples were digested with 0.01 unit of DNase I. In lanes 10−17, the nucleosomal DNA samples were digested with 0.1 unit of DNase I. The location of the Cbfα binding site is shown by a filled bar at the right of the gel. The CBF factors present in each binding reaction are indicated at the top of the gel. A schematic representation of the assembled segment including cleavage sites for retriction enzymes is also shown at the top. (B) −207/−56 OC promoter segment as naked DNA (lanes 1−6) or reconstituted nucleosome (lanes 7−12) was incubated with the restriction enzymes *Pst*I (upper panel) or *Fsp*I (lower panel) for 20 min at 30 °C as described under Materials and Methods. The digestions were performed in the absence (lanes 2 and 7) or presence (lanes 3−6 and 8−12) of the CBF transcription factors added at a final concentration of 300 nM. The percentage of fragment cleaved is shown below each lane. Lane 1 = undigested DNA, lane 2 = digested DNA, lane 3 = DNA+GST-Cbfα2A, lane 4 = DNA+GST-Cbfα2A/Cbfβ, lane 5 = DNA+GST-Cbfα2B, lane 6 = DNA+GST-Cbfα2B/Cbfβ, lane 7 = digested nucleosome, lane 8 = nucleosome+GST-Cbfα$_{50-175}$, lane 9 = nucleosome+GST-Cbfα2A, lane 10 = nucleosome+GST-Cbfα2A/Cbfβ, lane 11 = nucleosome+GST-Cbfα2B, and lane 12 = nucleosome+GST-Cbfα2B/Cbfβ.

5A), 9 bp upstream (nucleosome b, Figure 5A), or immediately downstream (nucleosome c, Figure 5A) from the nucleosome edge. Nucleosome movements, especially upstream, would increase the chance of exposure of the Cbfα site and favor the binding of Cbfα2 proteins.

By using a previously described approach (*33*), the position of the nucleosomes reconstituted on the −287/−106 segment was determined. This technique involved digesting the reconstituted fragment with micrococcal nuclease and mapping the position of the approximately 150 bp micrococcal nuclease-resistant fragment by digestion with one or two restriction enzymes. Strikingly, mapping of the reconstituted segment −287/−106 showed that this fragment positions nucleosomes at two principal locations (Figure 5C, lanes 7−10). The major location has a 3′-boundary at approximately −138 (Figure 5C, lanes 8 and 9, see upper bands of 126 nt and 146/142 nt generated by cleavage with *Stu*I and *Pst*I, respectively), and the minor location has a 3′-boundary at aproximately −114 (Figure 5C, lanes 8 and 9, see lower bands of 102 nt and 122/118 nt generated by cleavage with *Stu*I and *Pst*I, respectively). These results indicated that following reconstitution of the −287/−106 segment, the nucleosome was preferentially positioned such that the Cbfα binding site was partially exposed (Figure 5C, see diagrams below).

When the binding of these transcription factors to −287/−106 nucleosomal DNA was evaluated, we found that Cbfα2A alone interacted with the CBFα site, although this interaction was not as strong as with naked DNA (Figure 5A, compare lanes 3 and 11). Interestingly, the interaction of Cbfα2A with nucleosomal DNA was enhanced by the Cbfβ subunit (Figure 5A, lane 12). DNase I footprinting analysis indicated that higher concentrations of Cbfα2A are required to interact with nucleosomal DNA than with naked DNA (Figure 5A, compare lanes 10 and 11). For example, a concentration of 75 nM, which was sufficient to bind strongly to naked DNA (Figure 5A, lane 2), was insufficient to produce a detectable footprint on the nucleosomal DNA (Figure 5A, lane 10). Concentrations of Cbfα2A above 150 nM (not shown) and especially above 300 nM (Figure 5A) produced strong protection to DNase I. These concentration values are within the range estimated to exist inside of the nucleus and are equivalent to those utilized in previous studies (*30*, *31*).

Cbfα2B alone was not capable of binding to this OC promoter segment assembled as a nucleosome (Figure 5A, lanes 14 and 15). However, heterodimerization with the Cbfβ subunit resulted in strong interaction of the complex with the CBFα site (Figure 5A, lane 16). As for Cbfα2A, the Cbfα2B/Cbfβ interaction with nucleosomal DNA required higher concentrations of both transcription factors.

We next determined if binding of Cbfα2A and Cbfα2B/Cbfβ to the −287/−106 DNA segment of the OC gene promoter organized as a nucleosome was associated with sliding of the histone octamer. This sliding could be favored by Cbfα2 factor binding and would necessarily require a change in the translational position of the nucleosome, which can be detected by assessing changes in the digestion pattern of specific restriction enzymes (*31*). Thus, histone octamer sliding upstream would induce an increase in accessibility to *Pst*I. As shown in Figure 5B, interaction between the −287/−106 fragment assembled as a nucleosome and Cbfα$_{50-179}$, Cbfα2A, or Cbfα2B/Cbfβ did not significantly alter the cleavage efficiency of the enzymes *Pst*I and *Stu*I. This result indicates that no substantial change in the position

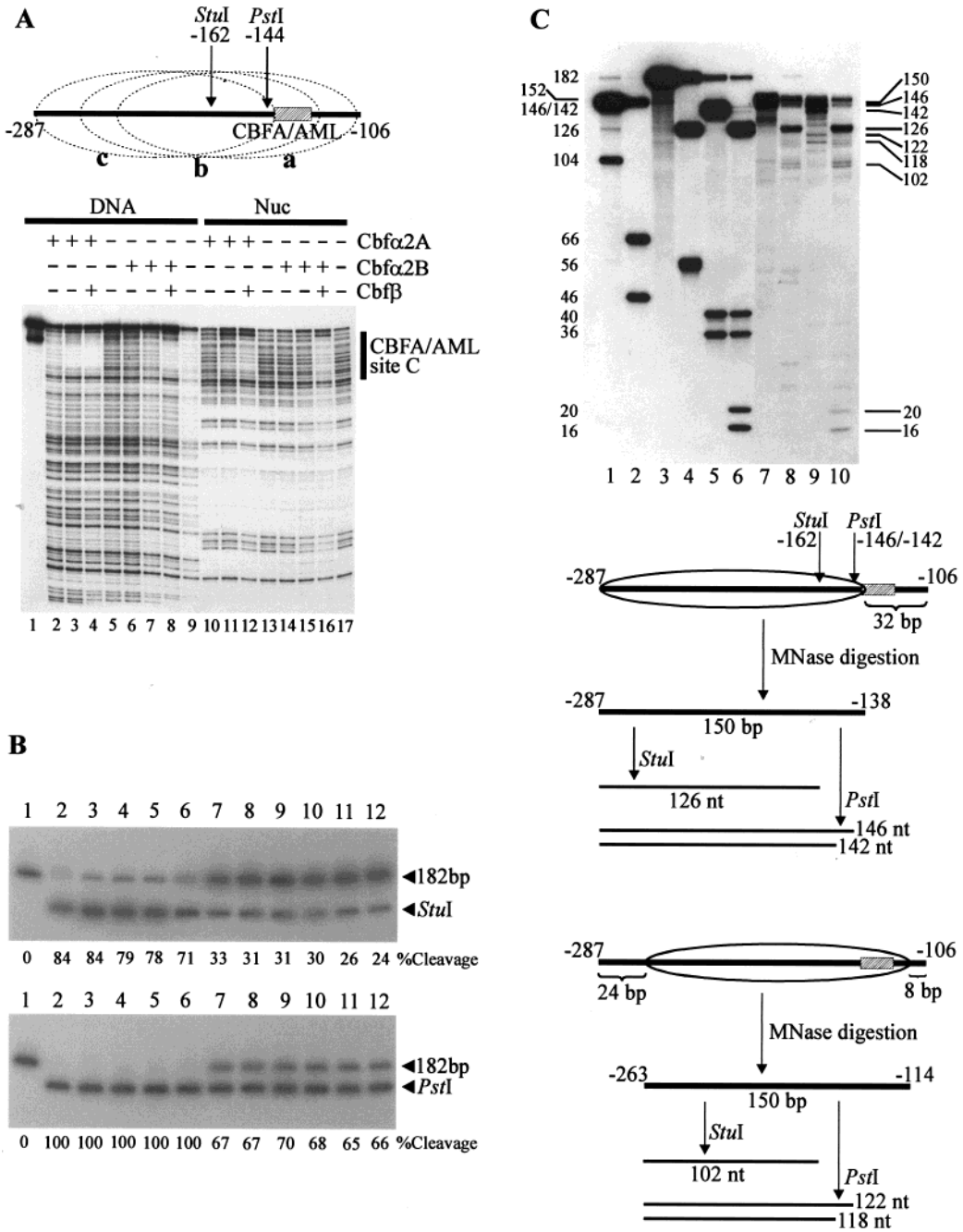

FIGURE 5: CBF transcription factor binding to the −287/−106 naked DNA fragment (lanes 1−9) or reconstituted as a nucleosome (lanes 10−17). (A) DNase I footprinting analysis. (B) *Stu*I and *Pst*I cleavage analysis. See legend in Figure 4 for an explanation of the symbols, the samples loaded in each lane, and the protein concentrations used. (C) Nucleosome positions along the −287/−106 segment were mapped as described under Materials and Methods. Cleavage products from the incubation of the approximately 150 bp core particle with *Stu*I were a major segment of 126 bp and a minor segment of 102 bp (lane 8). Similarly, cleavage with *Pst*I produced a major segment of 146/142 nt (cohesive end cleavage) and a minor segment of 122/118 nt. Diagrams representing the two principal positions adopted by the nucleosomes along the −287/−106 fragment are shown below the gel. Lanes 1−6 contain molecular size and specific restriction enzyme cleavage markers and lanes 7−10 the nucleosome mapping samples. Lane 1 = undigested −207/−56 DNA segment + −207/−56 segment digested with *Fsp*I; lane 2 = −207/−56 segment digested with *Pst*I + −207/−56 segment digested with *Stu*I; lane 3 = undigested −287/−106 segment; lane 4 = −287/−106 segment digested with *Stu*I; lane 5 = −287/−106 segment digested with *Pst*I; lane 6 = −287/−106 segment digested with *Pst*I and *Stu*I; lane 7 = core particle DNA fragment; lane 8 = core particle DNA digested with *Stu*I; lane 9 = core particle DNA digested with *Pst*I; lane 10 = core particle DNA digested with *Stu*I and *Pst*I.

of the histone octamer is induced by the binding of the CBFα factors. The absence of nucleosome sliding was also confirmed by nucleosome position mapping (data not shown).

Our results suggest that purified CBFα factors bind to the proximal OC gene promoter CBFα site assembled as a nucleosome only if the reconstituted DNA segment is 180 bp or longer (not shown). Assembly of these longer segments allows a degree of flexibility in the position of the histone octamer along the DNA fragment that results in exposure of the CBFα binding site. To further confirm this indication, we assembled the segment −257/−57 as a nucleosome and evaluated binding of CBFα factors by DNase I footprinting analysis. Our rationale was that after reconstitution of this 201 bp fragment, nucleosomes would block access to the

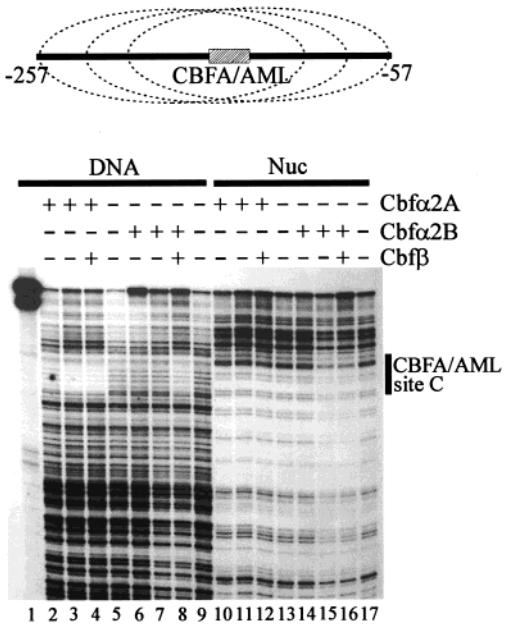

FIGURE 6: CBF transcription factors do not bind to the −257/−57 nucleosomal fragment. CBF transcription factor binding to the −257/−57 naked DNA fragment (lanes 1−9) or reconstituted as a nucleosome (lanes 10−17) analyzed by DNase I footprinting. See legend in Figure 4 for an explanation of the symbols, the samples loaded in each lane, and the protein concentrations used.

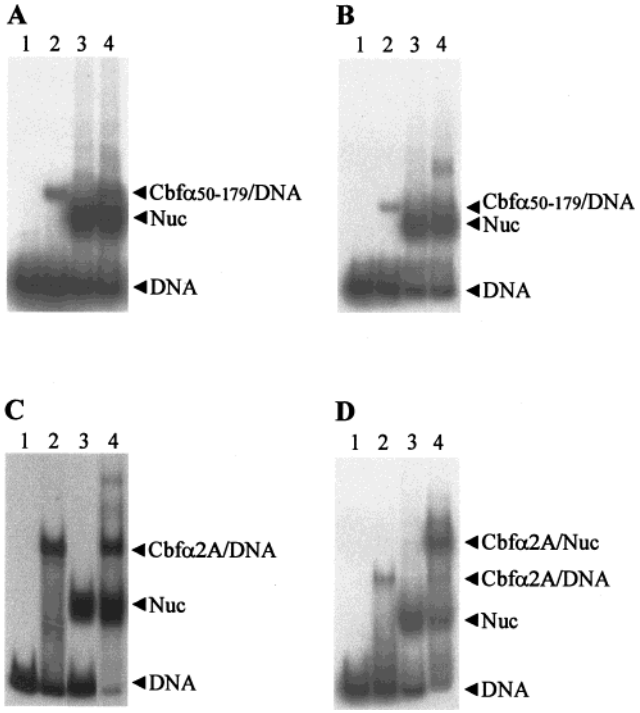

FIGURE 7: The *runt* homology domain is not sufficient to bind to nucleosomal DNA. Binding of $Cbf\alpha_{50-179}$ (A and B) and Cbfα2A (C and D) to naked or nucleosome-assembled OC promoter segments as analyzed by EMSA. (A and C) Segment −257/−106, (B and D) segment −287/−106. The positions of the free DNA, free nucleosome, Cbfα−DNA complexes, and Cbfα−nucleosome complexes are indicated at the right side of each gel. Lane 1 = DNA alone, lane 2 = DNA+GST-Cbfα, lane 3 = nucleosome alone, and lane 4 = nucleosome+GST-Cbfα.

CBFα site independent of the position adopted by the histone octamer along the segment (Figure 6, see diagram on top). As shown in Figure 6, neither Cbfα2A nor Cbfα2B/Cbfβ is able to interact with the −257/−57 fragment assembled as a nucleosome (Figure 6, lanes 10−17).

To determine if the *runt* homology DNA binding domain is sufficient to bind nucleosomal DNA, we carried out studies that allow us to compare the binding properties of Cbfα2A and $Cbf\alpha_{50-179}$. As shown in Figure 7, the *runt* homology domain present in $Cbf\alpha_{50-179}$ does not interact with a CBFα site when different segments of the OC promoter are assembled as a nucleosome. The presence of Cbfβ does not alter this result (data not shown). As Cbfα2A can bind to the −287/−106 segment organized as a nucleosome (Figure 7D, lane 4), it appears that protein segments adjacent to the *runt* homology domain are required for binding nucleosomal DNA.

Taken together, these results lead us to conclude that CBFα factors bind to those mononucleosomes reconstituted on the −287/−106 fragment that present the CBFα binding site partially exposed or in close proximity to the nucleosome edge. More interestingly, the preferential nucleosome positioning shown by the DNA sequence upstream of the rat OC promoter CBFα site may be responsible at least in part for the chromatin reorganization associated with OC gene transcription activation in vivo (see Discussion).

DISCUSSION

We reconstituted, into mononucleosomes, segments of the proximal promoter region of the rat OC gene which contain a high-affinity binding site for CBFα/AML transcription factors (*16*−*19*) and subsequently performed protein−DNA interaction studies within a chromatin-like context. We compared binding of purified recombinant Cbfα2A and Cbfα2B proteins. These two splice variants of the CBFα2 gene are both competent DNA binding proteins but represent transcriptionally active (Cbfα2B) and inactive (Cbfα2A) isoforms (*21*, *22*). In addition, we have assessed the influence of heterodimerization with the Cbfβ subunit on the ability of these two factors to bind to naked and nucleosomal DNA. Our results demonstrate that both Cbfα2A alone and Cbfα2B complexed with Cbfβ can interact with nucleosomal DNA only if there is a degree of flexibility in the positioning of the histone octamer on the DNA fragment and exposure of the CBFα site. This situation was achieved when we used a DNA segment of 182 bp (or longer, but not shown) from the rat OC promoter that preferentially positions mononucleosomes upstream of the CBFα binding site and leaves this element partially exposed. Neither Cbfα2B/Cbfβ nor Cbfα2A was able to bind if shorter OC promoter DNA fragments (152 bp) were assembled into nucleosomes or when a 201 bp segment that does not leave the CBFα binding site at the edge was analyzed.

Binding of Cbfα2A and Cbfα2B/Cbfβ to nucleosomal DNA required at least 2-fold higher concentration than was necessary for binding to naked DNA. These higher concentrations, however, lie within the range estimated to exist inside nuclei for several mammalian transcription factors (*30*, *31*).

Taking advantage of convenient restriction enzyme cleavage sites, we determined that binding of Cbfα2A or Cbfα2B/Cbfβ was not associated with a significant change in the translational position of the histone octamer. This conclusion is based on the fact that a marked movement of the octamer in the upstream direction would have resulted in increased

accessibility of the enzyme *Pst*I to its site, which lies immediately upstream of the Cbfα binding site (see Figure 5, top of the gels). This result has also been confirmed by nucleosome mapping (data not shown).

Previous studies have established that when nucleosomes are reconstituted on linear DNA fragments (longer than 150 bp), there is a greater preference for nucleosome location at the center and at the ends of the fragment (*35*). By mapping nucleosome positions, we determined that the major fraction of the nucleosomes reconstituted on the −287/−106 fragment was located at the 5′ end, leaving the CBFα site immediately at the edge and at least partially available for binding of Cbfα2A or Cbfα2B/Cbf*β*. On the other hand, the minor fraction of the nucleosomes was located at the 3′ end of the −287/−106 fragment, leaving the CBFα binding site aproximately 16 bp from the edge. Based on these results, we propose that binding of the Cbfα2A or Cbfα2B/Cbf*β* factors to the −287/−106 mononucleosome is principally due to interaction of these factors with the partially exposed CBFα site. Although from these data it is not possible to conclude exactly which nucleotides must be free in order for CBFα binding to occur, our results strongly suggest that nucleosomes positioned with their 3′ boundaries upstream of position −138 are capable of binding CBFα factors. Interestingly, DNase I footprinting analysis has shown that binding of purified CBFα factors to the proximal OC promoter CBFα site protects a DNA region beyond −138 (unpublished results). This indicates that binding of CBFα factors with the reconstituted −287/−106 segment may involve nucleotides located upstream of the nucleosome edge.

Taken together, these results suggest that nucleosome translational positioning can be a major determinant of the binding of CBFα factors to DNA assembled as nucleosomes. This situation could be significantly favored in vivo by the activity of chromatin remodeling complexes such as CHRAC or NURF (*8*, *9*).

Most members of the CBFα/AML/PEBP2α family of transcription factors function as heterodimers, comprising an α subunit that contacts the DNA and a *β* subunit which does not (*21*, *22*). It has also been found that the *β* subunit increases the DNA binding affinity of the α subunit (*23*, *24*). Here, we report that, within the context of the native rat OC gene promoter, Cbfα2A did not require the Cbf*β* subunit to interact specifically with the Cbfα site. In contrast, the longer isoform Cbfα2B showed significant interaction only in the presence of Cbf*β*. Our results were confirmed by protein−nucleosomal DNA interaction studies. We found that Cbfα2A can interact in the absence of Cbf*β* and that this binding was further enhanced by its presence. In contrast, Cbfα2B binding to a segment assembled as a nucleosome strongly depended on the presence of the Cbf*β* subunit.

Hirai and collaborators (*28*) have reported that Cbfα2A binds DNA with higher affinity than Cbfα2B. They have also suggested that Cbfα2B may contain domains that dampen the binding potential of the *runt* domain. Recently, Ito and colleagues (*20*) have found that in the Cbfα2B/PEBP2α protein the DNA binding domain (*runt* homology domain) is intramolecularly masked. The region that seems responsible for this blocking resides within aa 183−291 (the NRDB, for negative regulatory region of DNA binding). These authors proposed that association of the Cbf*β* subunit with the *runt* domain causes a conformational change in the α subunit that exposes the *runt* domain and allows strong DNA binding. Cbfα2A originates in vivo by alternative splicing (*22*) which results in a truncated isoform that contains only 250 aa (see diagram in Figure 3A). Thus, a significant portion of the NRDB domain is missing in the Cbfα2A protein. Taken together, these results suggest that constitutive exposure of the *runt* domain in Cbfα2A allows a strong and Cbf*β*-independent interaction with DNA. However, binding to nucleosomal DNA not only involves the *runt* DNA binding domain, but also requires regions of the protein which are present in Cbfα2A but absent in $Cbf\alpha_{50-179}$. Thus, $Cbf\alpha_{50-179}$ was unable to interact with nucleosomal DNA even at significantly higher concentrations (Figure 7).

Transcription of the OC gene 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; *13*, *14*). The DNA segment between these two hypersensitive sites is organized as a nucleosome. It has been suggested that the translational position of this nucleosome might reflect protein−DNA interactions occurring in the proximal promoter region, that account for both formation of the proximal hypersensitive site and OC gene transcriptional activity (*14*, *15*). We have recently found that mutations that eliminate CBFα binding sites in the OC gene promoter result in a dramatic decrease in the transcriptional activity controlled by this promoter and in the absence of DNase I hypersensitivity (*36*). In addition, by combining nuclease digestion and ligation-mediated PCR analysis, we have observed that proteins interacting in vivo within the proximal region (−160 to −120) of the OC gene promoter appear to establish a downstream boundary for a nucleosome positioned immediately upstream (unpublished results). Taken together, these results suggest that the binding of CBFα transcription factors to the proximal promoter region may represent a key molecular event in the process that leads to OC gene transcriptional activation in vivo. Moreover, our results indicate that interaction of CBFα factors with chromatin may require a previous or concomitant nucleosomal remodeling step that exposes the binding site.

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