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# Nucleosome assembly on the human c-*fos* promoter interferes with transcription factor binding

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## ABSTRACT

**cAMP-responsive-element (CRE)-binding factors interaction with nucleosomal DNA has been investigated *in vitro* on the human c-*fos* promoter. Analysis of nucleosome reconstitution of this promoter shows a preferential nucleosome positioning on the proximal promoter sequences, including the CRE centered at -60 relative to the start site of transcription. CRE-binding protein (CREB) and modulator protein (CREM) are unable to interact with their recognition site incorporated in a nucleosome. However, competition between transcription factor binding and nucleosome assembly allows CREM binding and induces important modifications in the nucleosomal structure suggesting the displacement of nucleosomes. These findings imply that binding of transcription factors to the CRE prior to cAMP induction might be required to prevent the incorporation of this element in a nucleosome.**

## INTRODUCTION

In eucaryotes, gene transcription is the result of a complex interplay between transcription factors and chromatin. The interaction of *trans*-acting factors with specific DNA sequences, which is a prerequisite for the transcription process, must be achieved in a chromatin environment (1–3). The packaging of DNA into nucleosomes severely restricts the access of factors to their binding sites. The DNA wrapped in approximately two turns around the histone core (one tetramer of H3/H4 and two dimers of H2A/H2B) is highly bent and the access on one side of the double helix is occluded because it faces the histone octamer (4). Thus, factor binding to nucleosomal DNA may depend on the way the sequence is incorporated into the nucleosomes. Both the position of the boundaries of the nucleosome on the DNA (translational positioning) and the orientation of the double helix around the octamer surface (rotational positioning) are important determinants for nucleosome factor binding (5,6). Specific nucleosome organization has been shown to be a common feature among promoter regions and has been found to be critical for proper gene transcription by providing a defined architectural conformation for transcription to take place (7,8).

Binding of transcription factors to chromatin can be achieved in different ways. Binding sites may be left free of histone–DNA interactions by being located in a linker region between nucleosomes (9–11). Some transcription factors, including the glucocorticoid receptor (12–14), GAL4 (15,16) and Max (17), have been found to be able to bind DNA within the nucleosome, forming a ternary complex. In some cases, histone modifications such as acetylation appear to facilitate protein interaction with nucleosomal DNA (18). *Trans*-acting factor binding can also disrupt nucleosomes in order to permit stable binding for itself or for other factors (16,19). There are however, a number of examples where nucleosomes have been found to inhibit *trans*-acting factors binding and/or repress transcription (20–22). In some instances, access to DNA can only be gained after remodeling of the chromatin structure which may be induced by other *trans*-acting factors or by chromatin-remodeling factors such as the SWI/SNF complex (23). Other studies have demonstrated that the inhibition of transcription factor binding by chromatin can be prevented if transcription factors interaction with DNA precedes nucleosome assembly. Thus, DNA replication might provide an opportunity for a number of factors to access their sequences before it is packaged into chromatin (24–26). Recent evidence suggests that promoters can be preset for rapid induction of transcription as a result of a combined interaction of transcription factors and nucleosomes following DNA replication (27).

The c-*fos* proto-oncogene belongs to the class of 'immediate early genes' which are involved in converting extracellular and intracellular signals into changes in gene expression. c-*fos* transcription is induced rapidly and transiently by a variety of stimuli such as phorbol esters, growth factors, neurotransmitters, cAMP and others (28). A number of *cis*-acting elements have been defined in the promoter region, which allow activation or repression of transcription by distinct signal transduction pathways. The SRE (Serum Response Element) centered at position –300 with respect to the transcription initiation site is the molecular target of the protein kinase C (PKC) signal transduction pathway through binding of the SRF (Serum Response Factor) and

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associated factors (29). c-*fos* transcription is induced by the cAMP/PKA pathway through a major cAMP element (CRE) centered at –60 (30, 31). This element is recognized by the protein CREB (CRE-binding protein) and a family of structurally related factors referred to as the CREB/ATF family (32). These factors belong to the basic-leucine zipper (b-Zip) class of transcription factors and bind CRE as homo- or heterodimers. A recently cloned member of this family, CREM (CRE modulator) generates both activators and repressors of transcription through alternative splicing (33).

Distinct signal transduction pathways activate c-*fos* transcription independently of protein synthesis within minutes of exposure to activators (28). Interestingly, the c-*fos* regulatory elements appear to be constitutively occupied *in vivo*, suggesting that the c-*fos* promoter might be organized in a preset chromatin structure to allow a rapid response to stimuli (34,35). To understand the molecular basis of c-*fos* regulation, it is essential to determine how nucleosomes are organized on this promoter and how this structure affects the binding of regulatory factors. We have addressed this question by analyzing the nucleosome organization on the human c-*fos* promoter in an *in vitro* reconstitution system.

We present evidence that the proximal region of the c-*fos* promoter contains DNA sequences capable of positioning a population of rotationally phased nucleosomes *in vitro*. Pre-bound nucleosomes inhibit CREB and CREM binding to the CRE. However, concomitant incubation of transcription factors and histones during the nucleosome reconstitution process allows *trans*-acting factor binding and subsequent remodeling of the nucleosomal structure. These findings suggest that the formation of a pre-bound transcription factor complex during chromatin assembly could be a necessary step to mediate a rapid response to cAMP induction.

## MATERIALS AND METHODS

### DNA fragments

The 465 bp fragment used in reconstitutions contains the human c-*fos* promoter sequences –404/+42 and was obtained by a *Sst*II–*Bgl*II digestion of plasmid FC-3 (36). A –222/+42 fragment obtained by PCR was subcloned into the *Bam*HI–*Eco*RI sites of plasmid pUC19 to give pF2. A 264 bp *Eco*RI–*Bam*HI fragment (–222/+42) and a 234 bp *Eco*RI–*Pst*I fragment (–222/+12) derived from pF2 were used in reconstitution experiments. Fragments were end-labeled using the T4 DNA polynucleotide kinase (New England Biolabs).

### Nucleosome reconstitution

Purified core histones were prepared from chicken erythrocyte nuclei using hydroxylapatite chromatography (18,37). Nucleosomes were reconstituted by salt/urea dialysis as described (18) in 200 µl total volume. The final DNA concentration was 20 µg/ml and the histone:DNA (mass to mass) ratio was 0.8:1 (a 1:1 ratio corresponds approximately to one nucleosome core particle per 160 bp of DNA). The assembly process was 90% efficient as detected by mobility shift assay. In the case of co-reconstitution with CREB and CREM proteins (or control extract), 5 µg of proteins were simply added to the initial histone–DNA mix and the reconstitution conditions were kept identical.

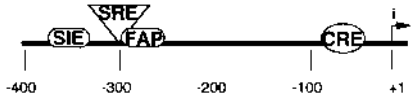


**Figure 1.** Schematic representation of the human c-*fos* promoter. The major *cis*-acting elements and their relative position to the start site of transcription are shown. Abbreviations: SIE, Sis Inducible Element; FAP, c-*fos*/AP-1 site.

### Production and binding of CREB/CREM proteins

pETCREB and pETCREM constructs have already been described (38). Bacterial extracts were prepared exactly as described (38). Protein concentration was determined by Biorad assay and the proteins were visualized on polyacrylamide–SDS gel. As reported, the recombinant proteins consisted of >80% of the total protein lysate (38). Binding assays were done in 25 mM Tris, pH 7.5, 6 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol 10% glycerol in 20 µl final volume for 15 min at 20°C.

### Footprinting

Hydroxyl radical footprinting was performed as described (18). For DNase I analysis, 1 mM $MgCl_2$ (final concentration) was added to 20 µl samples and digestion was carried out for 2–3 min at 20°C with 0.01 mg/ml DNase I (Sigma) for naked DNA and with 0.1 mg/ml DNase I for reconstitutes. Exonuclease III digestions were performed in 20 µl reactions in 10 mM Tris, pH 7.5, 25 mM KCl, 5% glycerol, 1 mM dithiothreitol for 15 min at 20°C. For naked DNA samples, 2–20 U of *Exo*III (Promega) were added as 20–200 U were used to digest reconstitution samples. Footprinting experiments were analyzed on 6% polyacrylamide gels containing 7.5 M urea.

## RESULTS

### Nucleosome positioning on the human c-*fos* promoter

A radiolabeled fragment from the human c-*fos* promoter spanning from –404 to +42 (relative to the start of transcription) was used to examine the organization of nucleosomes after *in vitro* reconstitution with purified histones (Fig. 1). This DNA fragment contains all the known c-*fos* regulatory elements (28). Nucleosomes were assembled by high-salt/urea dialysis at moderate (0.8:1 histones:DNA by mass) densities of nucleosomes to avoid non physiological close-packing of nucleosomes. Under these conditions two nucleosomes are expected to form on the DNA fragment. Assemblies were analyzed by mobility shift assays to verify the presence of retarded complexes (data not shown).

Analysis of reconstitutes by DNase I and OH-radical footprinting was performed to identify the positioning of nucleosomes on the promoter. Such a feature would be detectable by a periodicity of cleavage reflecting a periodic narrowing of the minor groove as a consequence of the wrapping of the DNA around the histone core (39). Rotationally positioned nucleosomal DNA will therefore generate a 10–11 bp periodicity of cleavage when cut by these two methods. Both analysis showed a typical 10–11 bp cleavage repeat pattern marking the presence of a positioned nucleosome on the proximal region of the promoter on the assembled template (Fig. 2A). Such a pattern was not observed on the distal part of the promoter. However, modifications of the cleavage pattern as compared to naked DNA could be detected indicating the

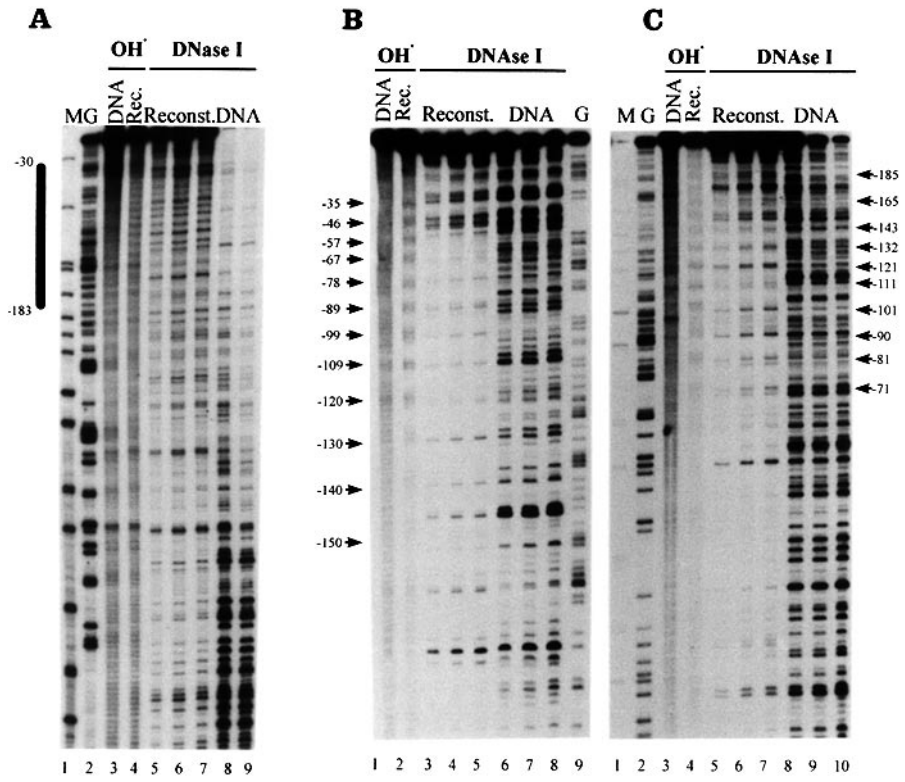


**Figure 2.** Footprints of the nucleosome-reconstituted c-*fos* promoter. **(A)** DNase I and hydroxyl radical analysis of the c-*fos* –404/+42 fragment labeled at the *Sst*II site (upper strand). Naked DNA (lanes 3, 8 and 9) or reconstitutes (lanes 4–7) were digested by hydroxyl radical (lanes 3 and 4) or with increasing amounts of DNase I (lanes 5–9). Lane G is a Maxam–Gilbert G-sequencing reaction. M is a *Hpa*II digest of pBR322. The solid bar indicates the region covered by the nucleosome. **(B)** Analysis of nucleosome reconstitutions on the c-*fos* –222/+12 fragment labeled at the *Eco*RI site (upper strand). Hydroxyl radical analysis is shown in lanes 1 and 2 and DNase I analysis in lanes 3–8. The free DNA cleavage pattern is shown in lanes 1 and 6–8. Digestion of the reconstitutes is shown in lanes 2–5. The G lane is as in (A). The arrows indicate the preferential cleavage sites in the reconstitutes. **(C)** Analysis of nucleosome reconstitutions on the c-*fos* –222/+42 fragment labeled at the *Bam*HI site (lower strand). Hydroxyl radical is shown in lanes 3 and 4 and DNase I analysis is shown in lanes 5–10. Naked DNA is shown in lanes 3 and 8–10. Nucleosome reconstitution digests are shown in lanes 4–7. Lanes M and G are as in (A).

presence of protein–DNA interactions. Analysis of the lower strand showed a similar digestion pattern (data not shown).

To analyze in more detail the nucleosome positioning on the proximal promoter region, we reconstituted nucleosomes on a smaller fragment containing the proximal sequences up to –222. OH-radical and DNase I analysis confirmed the presence of a clear 10–11 bp modulation of cleavage suggesting the presence of a positioned nucleosome between –35 and –185 (Fig. 2B and C). This periodic modulation of cleavage was observable on both DNA strands although the lower strand DNase I pattern was less apparent, presumably because of the sequence preference of the enzyme.

DNase I and OH-radical analysis give information on the rotational phasing of nucleosomes but do not give precise information on the boundaries of the nucleoprotein complex. Exonuclease III (*Exo*III) was therefore used to locate the extremities of the nucleosome on the proximal promoter DNA fragment (13). This enzyme, which progressively digests DNA through the 3′ end of the double helix until it encounters a physical barrier is a useful tool to determine nucleosome extremities (10,13). Analysis of both DNA strands revealed the presence of multiple barriers to the nuclease activity suggesting the presence of four nucleosome populations (Fig. 3). We believe this result reflects the presence of different populations of nucleosomes and not the 'read-through' of the enzyme in a single nucleosome since overdigestion of the samples did not produce additional signals but lead to the degradation of the pre-existing exonuclease stops (data not shown). Moreover the DNA size between the boundaries on either sides (~160 bp) corresponds to the length of DNA incorporated into a nucleosome.

Taken together these results indicate that the c-*fos* promoter contains a strong nucleosome-positioning element which directs *in vitro* the formation of four nucleosomes with different translational positioning but identical rotational settings (Fig. 4A). The cAMP-responsive element (CRE) centered at –60 is included in the nucleosomes. Analysis of the rotational setting of the DNA around the histone octamer (Fig. 4B) shows that more than half of the CRE consensus sequence is located in major grooves facing towards the histone core. As the CRE element has been shown to be responsible for the induction of c-*fos* transcription by the cAMP pathway (30,31), we wished to determine how the CRE-binding proteins interact with this sequence in presence of a nucleosome.

## Interaction of CRE-binding proteins with nucleosomal DNA

Several members of the CREB/CREM family of factors were bacterially expressed in pET vectors as previously described (38) and tested for their ability to bind free or reconstituted c-*fos*

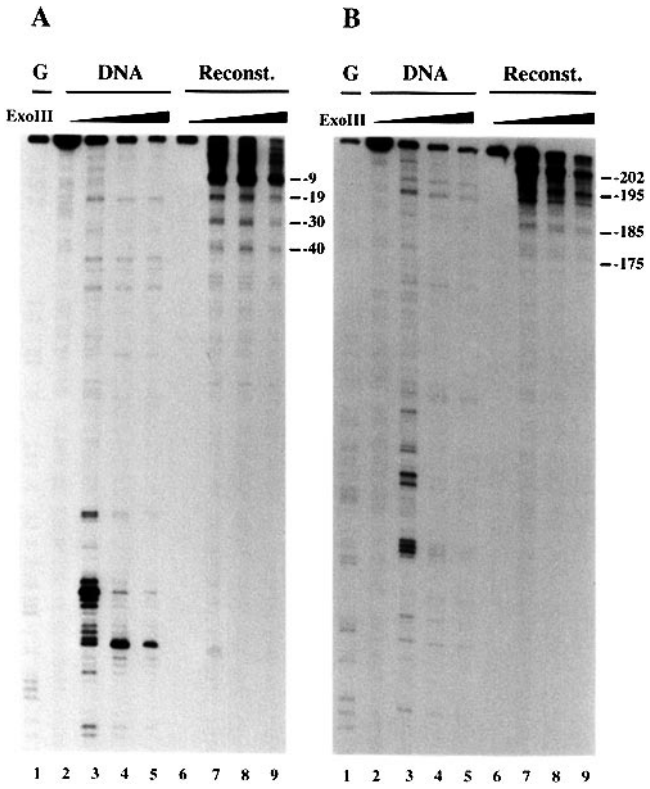


**Figure 3.** Exonuclease III analysis of the reconstituted c-*fos* proximal promoter fragment. **(A)** The c-*fos* –222/+12 fragment was labeled at the *Eco*RI site. Free DNA (lanes 2–5) or reconstitutes (lanes 6–9) were digested with increasing amount of enzyme as indicated in the materials and methods. The *Exo*III-resistant borders are indicated. G is a Maxam–Gilbert sequencing reaction showing the guanines of the sequence. **(B)** The c-*fos* –222/+42 fragment was labeled at the *Bam*HI site. Naked DNA (lanes 2–5) or reconstitutes (lanes 6–9) were digested with increasing amount of enzyme. Lane G is as in (A).

promoter fragments. As seen by DNase I footprinting, bacterially expressed CREB, CREMβ and CREMτ bind DNA efficiently at the CRE element as seen by the clear protection obtained on this element (Fig. 5A, lanes 3–5). However, incorporation of the CRE in a nucleosome prevents interaction of the factors with their recognition site even when high amounts of proteins (20-fold excess) were added (Fig. 5A, lanes 8–13). Exclusion from the nucleosomes was complete since mobility shifts also failed to detect any binding of CRE proteins on the reconstituted nucleosomes (data not shown). We obtained similar results with other CREM proteins [CREMα and ICER (40), data not shown]. We conclude that the presence of positioned nucleosomes on the CRE prevents interaction of *trans*-acting factors.

It has been suggested that in order to interact with their recognition sequences some transcription factors should bind to promoter sequences before nucleosomes assemble on the DNA (21,24). Thus, we postulated that the inability of CREB/CREM factors to interact with their recognition site included in a nucleosome could be alleviated if binding of the factors could precede the formation of the nucleosomes. We performed reconstitutions to analyze if CREB/CREM proteins would be able to bind DNA in the conditions used for nucleosome reconstitutions. CREMβ was incubated with the 264 bp fragment of the c-*fos* promoter and subjected to high salt/urea dialysis. DNase I analysis of the resulting sample showed that CREMβ was bound the CRE element at the end of the reconstitution procedure (Fig. 5B, lane 4, see also Fig. 5C, lane 3). Remarkably, when histones were co-incubated with CREMβ during the reconstitution procedure, both the protection on the CRE and the nucleosome 10–11 bp repeat pattern were observed (Fig. 5B, lane 6). No modification was observed in the rotational phasing of the nucleosomes but on the lower DNA strand, the protection observed over the CRE appears to extend in the 5′ region below this element (Fig. 5B, lane 6). Some changes in the DNase I cutting pattern were also observed in the 25 bp upstream of the CRE. Whereas equivalent amount of CREM were used in reconstitutions in presence or absence of histones, these modifications of DNAse I cleavage

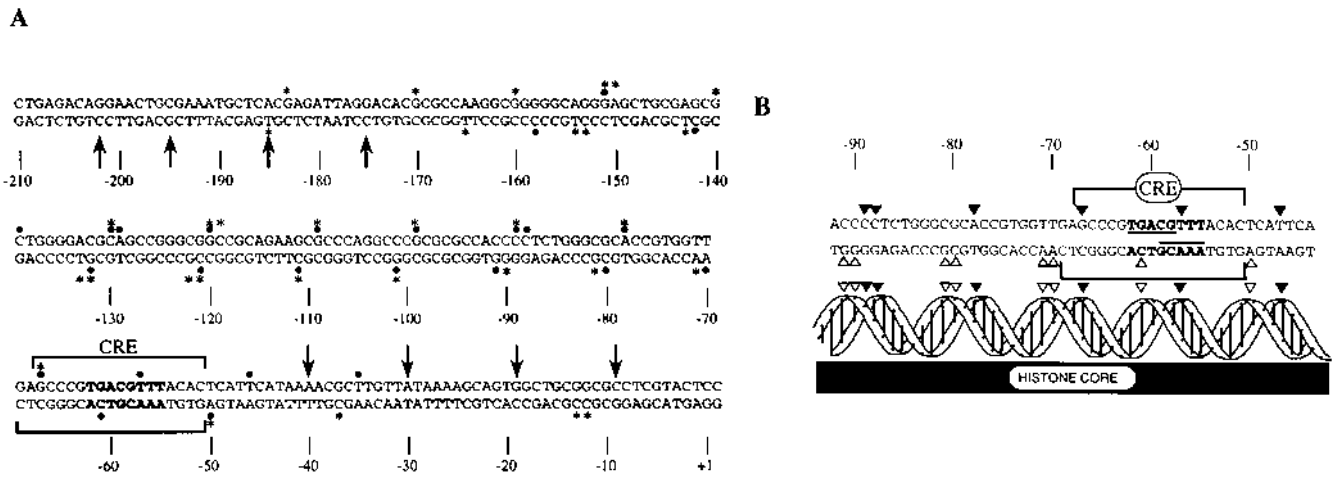


**Figure 4.** Summary of the nucleosome positioning analysis. **(A)** The nucleotide sequence of the c-*fos* promoter between –226 and +2 relative to the transcription initiation site is shown. DNase I preferential cleavage sites on the nucleosome-reconstituted fragments are indicated by asterisks, hydroxyl radical by black dots and *Exo*III by arrows. The position of the CRE is indicated. Bold letters outline the CRE consensus whereas brackets indicate the regions protected in DNase I footprinting analysis. **(B)** Representation of the helical setting of the DNA on the histone octamer. The DNase I and hydroxyl radical preferential cut sites on the top strand (filled triangles) and bottom strand (open triangles) are indicated. The filled bars indicate the major groove facing towards the histone core.

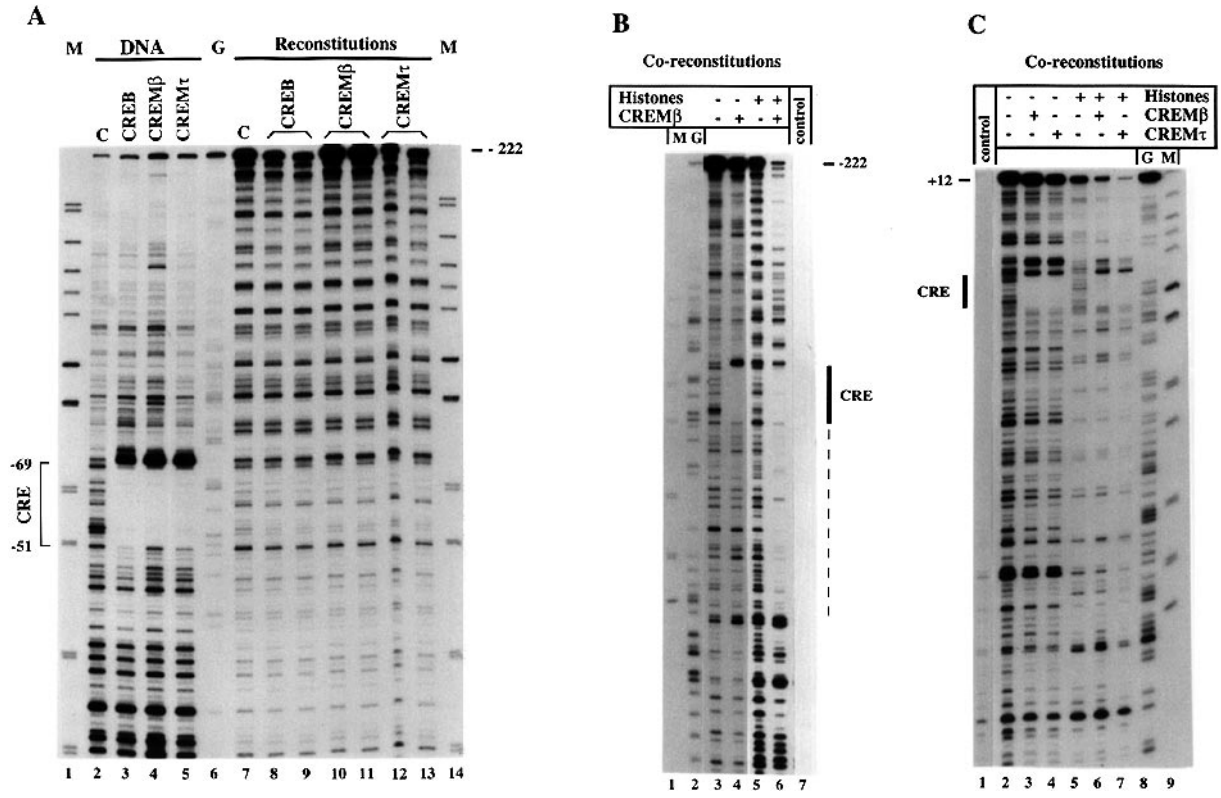


**Figure 5.** DNase I analysis of CREB and CREM binding after or during nucleosome assembly. **(A)** Analysis of CREB and CREM binding after nucleosome assembly on the c-*fos* –222/+42 fragment (lower strand). Free DNA (lanes 2–5) or reconstitutes (lanes 7–13) were incubated with bacterially produced CREB, CREMβ and CREMτ or with an untransformed bacteria control extract (C) as indicated. Naked DNA was incubated in presence of 0.2 µg of extract. Reconstitutes were incubated with 2 µg (lanes 7, 8, 10 and 12) or 4 µg (lanes 9, 11 and 13) of extract. M is a *Hpa*II digest of pBR 322. **(B)** Analysis of CREMβ binding during nucleosome assembly on the c-*fos* –222/+42 fragment (lower strand). Nucleosome reconstitutions were performed as described in Material and Methods with (lanes 5 and 6) or without (lanes 3 and 4) histones in presence (lanes 4 and 6) or absence (lanes 3 and 5) of CREMβ. In lane 7, radiolabeled free DNA was incubated with unlabeled reconstituted fragment and digested with DNase I as in lanes 5 and 6. Lane M is as in (A), lane G is a Maxam–Gilbert G-sequencing reaction. The protection over the CRE is indicated by the black bar. The dashed line outlines the extended protection observed in co-reconstitutions. **(C)** Analysis of CREMβ and CREMτ binding during nucleosome assembly on the c-*fos* –222/+12 fragment (upper strand). Reconstitutions were performed in presence (lanes 5–7) or absence (lanes 2–4) of histones. CREMβ was included in the reconstitution reactions analyzed in lanes 3 and 6 whereas CREMτ was added to the samples analyzed in lanes 4 and 7. Lanes M and G are as in (B). The control in lane 1 was prepared and digested as in lane 7B.

pattern were observed only when CREM was co-incubated with histones (Fig. 5B, compare lanes 4 and 6). Thus, these changes suggest a distortion of the nucleosomal structure due to the simultaneous interaction of the histones and CREMβ with the c-*fos* DNA. However, the possibility remained that the footprint on the CRE was due to the binding of CREMβ to nucleosome-free DNA molecules juxtaposing on the nucleosome pattern. A control experiment was performed to verify the absence of significant amounts of naked DNA in the reconstitution samples. Labeled naked DNA was mixed with nucleosome-reconstituted unlabelled templates and then digested with the amount of DNase I used to digest reconstitutes. Under these digestion conditions, naked DNA was digested to completion (Fig. 5B, lane 7, see also Fig. 5C, lane 1) which ruled out the possibility that the protection over the CRE could be due to CREMβ bound to unreconstituted templates. Rather, this result suggests that co-incubation of histones and CREMβ can lead to the simultaneous binding of CREMβ and nucleosomes on the promoter. DNase I footprinting analysis of CREM/histones co-reconstitutions on the upper strand confirmed the co-existence of CREMβ and nucleosome complexes on the promoter (Fig. 5C, lane 6). CREMτ also appears to be able to interact in the same manner as CREMβ in presence of nucleosomes as both cleavage patterns appear very similar (Fig. 5C, lanes 6 and 7). The presence of CREM did not affect the nucleosomal pattern on the upper strand as observed on the lower strand.

To confirm the binding of CREMβ and nucleosomes on the same DNA molecules, we analyzed the co-reconstitutions shown in Figure 5 by gel mobility shift assay (Fig. 6). When CREMβ was incubated with the c-*fos* fragment several complexes were observed. At the high concentration of protein used in these experiments CREMβ forms multimers on the c-*fos* promoter fragment, the faster migrating complex corresponding to a single dimer of CREMβ as determined by factor titration experiments (Fig. 6, lane 3 and data not shown). In contrast, the nucleosome-reconstituted fragment migrated as a single major complex (Fig. 6, lane 2). Co-reconstitution of CREMβ and histones resulted in the shifting of part the octamer complex into larger complexes which clearly differed from those observed when CREMβ was bound to naked DNA (Fig. 6, lane 4). This analysis confirmed the binding of both nucleosomes and CREMβ on the same DNA fragment.

The binding of CREM in reconstituted templates on sequences normally included in the nucleosomes suggested that rearrangements must have occurred which could not be detected by DNase I analysis. First, we confirmed that CREM binding was not due

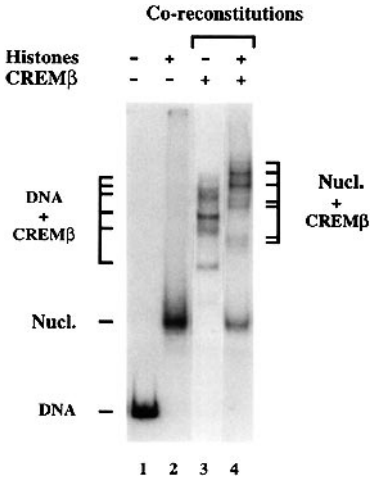


**Figure 6.** Mobility shift analysis of co-reconstitutions with CREMβ. Reconstitutions were performed on the c-*fos* –222/+12 fragment in presence of either histones (lane 2) or CREMβ (lane 3) or with both histones and CREMβ (lane 4) as described in Materials and Methods. Aliquots were run on 3.5% acrylamide gel containing 0.5× TBE. Complexes resulting from the binding of CREMβ to DNA are shown on the left, complexes occuring upon binding of both CREMβ and nucleosomes are shown on the right.

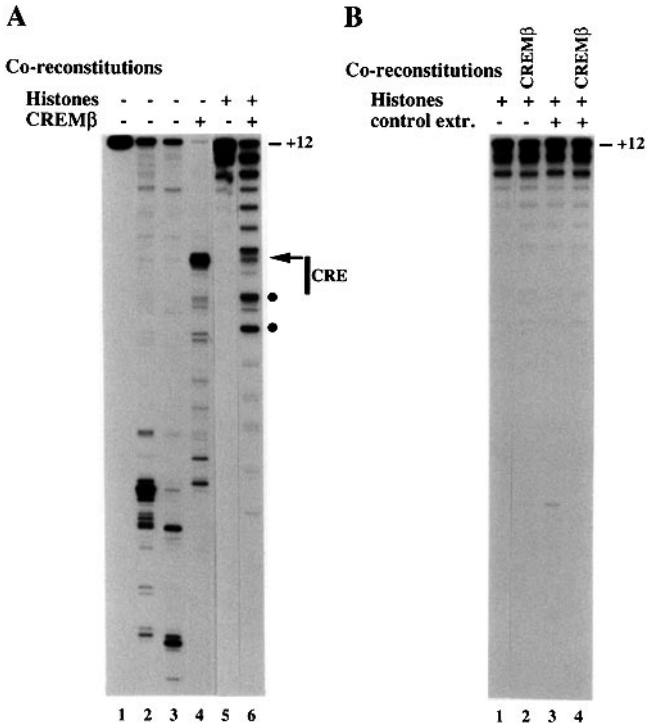


**Figure 7.** Exonuclease III analysis of CREMβ binding during nucleosome assembly. **(A)** The c-*fos* –222/+12 fragment was digested by *Exo*III after reconstitution in presence (lanes 5 and 6) or absence (lanes 1–4) of histones. CREMβ was included in the nucleosome assembly reactions analyzed in lanes 4 and 6. The arrow indicates the CREMβ-dependent *Exo*III stop. The position of the CRE is indicated by the black bar. The two new *Exo*III stops which are observed in the co-reconstitution sample are indicated by black dots. **(B)** The same fragment as in (A) was reconstituted into nucleosomes in presence (lanes 3 and 4) or in absence (lanes 1 and 2) of untransformed bacteria control extract. After reconstitution, aliquots in lanes 2 and 4 were incubated with 1 µg of CREMβ before being subjected to *Exo*III digestion.

to a partial degradation of the histones by potential nuclease activities present in the bacterial extracts during the reconstitution procedure by checking the integrity of the histones on SDS–PAGE (data not shown). Co-reconstitutions were then analyzed by *Exo*III cleavage. CREMβ binding to the CRE was clearly detected on naked DNA (Fig. 7A, lane 4). Co-reconstitutions of CREMβ and histones affected dramatically the nucleosome arrangement on the template as compared to the reconstitutions done in absence of the transcription factor (Fig. 7A, lanes 5 and 6). In particular, two new nuclease-resistant barriers were observed upstream of the CRE, one located at the boundary of the binding site, the other 10 bp further. This nucleosome rearrangement was specific to the presence of CREMβ (and CREMτ, data not shown) since co-reconstitutions performed with proteins from a control bacterial extract did not disturb the nucleosomal pattern (Fig. 7B, lane 3). Furthermore, the addition of CREMβ to samples reconstituted in presence of the bacterial control extract did not allow CREM binding nor produced any change in the nucleosomal pattern (Fig. 7B, lane 4 and data not shown). Thus, it appears that CRE binding and nucleosome rearrangements are only mediated by CREM.

## DISCUSSION

Our results show that CRE-binding proteins can successfully compete with histones for binding on the c-*fos* promoter. Preformed rotationally phased nucleosomes positioned on the c-*fos* proximal promoter prevent the interaction of CREB and CREM with their binding site. However, co-incubation of the *trans*-acting factors with the histones during the reconstitution process allows transcription factor binding to the CRE on the nucleosome-reconstituted promoter. We found that the nucleosomal organization was disturbed upon transcription factor binding, probably reflecting the displacement of the nucleosomes on sequences immediately adjacent to the CRE.

Nucleosomes can be specifically positioned by DNA sequences. Nucleosome positioning depends on DNA structural features such as flexibility or intrinsic curvature rather than precise sequence requirement (41). Nucleosome reconstitution on a DNA fragment comprising the entire c-*fos* promoter *in vitro* revealed a specific nucleosome positioning on the proximal promoter sequences but not on the distal part of the promoter. Under the conditions of reconstitutions used which allow the formation of two nucleosomes per DNA template, we conclude that the proximal part of the promoter contains sequence-specific nucleosome positioning signals which direct preferential nucleosome assembly whereas on the distal part, the nucleosomes are randomly arranged. Further analysis using DNase I, OH-radical and *Exo*III cleavage on a smaller DNA fragment comprising the proximal promoter sequences suggested the presence of four nucleosomes with identical rotational settings but translationaly staggered by one helical repeat. Such multiple nucleosome positioning has previously been reported for other DNA sequences *in vitro* and *in vivo* (42–45). In the absence of translational signals such as specific DNA structural features or linker histones, nucleosomes have been shown to adopt different translational positions possibly by being mobile (43–45).

The major consequence of this multiple positioning on the c-*fos* promoter is that the CRE is incorporated in all four translational positions. The single rotational phasing directs the CRE major groove towards the histone core, preventing the interaction of CREB and CREM. Thus, the minimum requirement for these factors to bind may not accommodate to the constraints imposed on the DNA in a nucleosome. The CRE 8 bp palindromic sequence is recognized by the basic region adjacent to the leucine-zipper such that the two positively-charged α-helices are in contact with the two halves of the palindrome in the major groove of the DNA helix (46). As a result, the CRE is slightly bent towards the leucine-zipper. In addition, flanking bases on each side of the core 8 bp sequence appear to be important for CREB binding (47). Thus, it seems unlikely that CREB or CREM can interact with their site in a nucleosome in any rotational position, although this remains to be tested. Phosphorylation of CREB and CREM by PKA has been reported to have a positive effect on DNA binding although this remains controversial (32). We found that the phosphorylation of CREB and CREM by PKA had no effect on the interaction of these factors with nucleosomal DNA (data not shown). Thus, an alternative for CREB/CREM binding to the CRE would be to interact before nucleosome assembly.

The addition of CREM in the reconstitution assays resulted in the concomitant binding of the transcription factor and the nucleosomes. This was observed in three different assays. First, in DNAse I footprinting experiments, we observed both the protection on the CRE and the nucleosomal cleavage pattern indicating the binding of CREM in presence of nucleosomes. Second, in mobility shift assays, co-incubation of CREMβ and histones resulted in the appearance of larger complexes than reconstitutions done in presence of either CREMβ or histones alone, confirming the interaction of CREM on the nucleosome-reconstituted c-*fos* promoter. Finally, as seen by the presence of new *Exo*III-resistant boundaries upstream of the CRE, it appeared that an important remodeling of the nucleosomal structure had occurred upon CREM binding. This could be explained either by a destabilization of the nucleosomes by the CRE-bound factors allowing the *Exo*III to penetrate in the nucleosomal structure, or by a displacement of the nucleosomes on new translational positions immediately upstream from this element. However, the size of the CREM dimers (CREMβ, 66 kDa; CREMτ, 84 kDa) and the tight contacts they make with DNA argues against their incorporation in a nucleosome. Therefore, this result more likely reflects the displacement of nucleosomes onto new translational positions, leaving the rotational phasing unchanged. Earlier studies have documented nucleosome positioning by specific DNA binding proteins. Specific interaction of proteins with DNA was shown to generate arrays of positioned nucleosomes whereas a random organization was observed in absence of factor binding (48–50). However, in the case of the c-*fos* promoter described here, nucleosomes were already positioned in the absence of CRE-binding proteins. Thus, it appears that *trans*-acting factors interaction with the CRE could provide a new boundary on pre-existing sequence-dependent nucleosome positioning signals.

Nucleosome assembly is a stepwise reaction involving a first interaction of the H3/H4 tetramer and the subsequent addition of two H2A/H2B dimers (51). The association of H2A/H2B has been shown to be inhibitory to some *trans*-acting factors binding (reviewed in 8). The structure of the nucleosome is known to change dramatically with ionic environment consistent with an increasingly relaxed secondary structure as the salt concentration is raised (52,53). We did not detect any interaction of CREB/CREM after reconstitution of the c-*fos* promoter with H3/H4 tetramers (data not shown). Thus, the interaction of CRE-binding factors during the reconstitution process is likely to precede the association of histones with DNA or could occur at intermediate ionic strength (0.2–0.7 M NaCl) when the histone–DNA interactions can be easily disrupted.

As stable nucleosomes restrict the access to the CRE, interaction of CREB/CREM *in vivo* with their recognition site could be mediated by a disruption of the nucleosomal structure. A number of factors have been shown to destabilize and remodel nucleosomes, promoting trans-acting factors binding. One is the SWI/SNF complex, originally found in yeast (54) but for which a homolog has also been identified in human cells (55). This complex appears to interact with nucleosomal DNA and alter histone–DNA contacts, assisting transcription factor binding (23). NURF, another factor recently purified from *Drosophila* is able to alter nucleosomal arrays and facilitate the interaction of the GAGA transcription factor (56). Also, a yet unidentified factor from *Drosophila* embryos has been reported to promote chromatin reorganization (57). It would be interesting to determine if these factors are able to disrupt the positioned nucleosomes on the c-*fos* promoter to allow CREB/CREM binding after nucleosome assembly.

Alternatively it is possible, as it has been suggested earlier (24,25,58), that CRE-binding proteins interact with the CRE following DNA replication by competing with the histones for binding to DNA. Although there is, at the moment, no information about the organization of the nucleosomes and transcription factors on the c-*fos* proximal promoter sequences *in vivo*, there is evidence that CREB-related proteins might be bound to the CRE before cAMP induction. Earlier studies have suggested that the c-*fos* CRE might be constitutively occupied *in vivo* in various mouse tissues and in a human cell line (34). Also, on the phosphoenolpyruvate carboxykinase (PEPCK) gene, the CRE site is occupied by CREB before induction by cAMP suggesting that phosphorylation of CREB by PKA occurs on dimers already bound to their site (59). Similarly, two AP-1 binding sites on the c-*jun* promoter appear constantly occupied *in vivo*, independently of phorbol ester stimulation or UV irradiation (60). The SRE on the c-*fos* promoter has also been found occupied by a complex of transcription factors before induction by epidermal growth factor and these protein–DNA contacts remain unchanged during gene activation and subsequent repression (35). Thus, the pre-establishment of protein–DNA complexes before induction might be a common mechanism for immediate early genes for rapid and transient response to extracellular signals. Our findings suggest that CREB/CREM binding prior to nucleosome assembly might be a necessary step to prevent a negative regulation by chromatin and to provide a defined nucleosomal organization for transcription to take place.

A recent investigation of the regulation of c-*fos* transcription in transgenic mice have suggested that the regulatory elements in c-*fos* function interdependently, as point mutations in any one of the SIE, SRE, FAP or CRE sequences abolished transcription from the c-*fos* promoter (61). Again, this observation implies that the regulatory elements are constitutively occupied and suggests that induction *in vivo* requires a sophisticated arrangement of several transcription factors. Interestingly, a nucleosome positioned between the CRE and the FAP site (see Fig. 1) could bring the CRE and the upstream regulatory elements fairly close together.

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