CBF/NF-Y Functions Both in Nucleosomal Disruption and Transcription Activation of the Chromatin-assembled Topoisomerase IIα Promoter

TRANSCRIPTION ACTIVATION BY CBF/NF-Y IN CHROMATIN IS DEPENDENT ON THE PROMOTER STRUCTURE*

Received for publication, July 23, 2001, and in revised form, August 15, 2001
Published, JBC Papers in Press, August 20, 2001, DOI 10.1074/jbc.M106918200

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To understand the role of CCAAT-binding factor (CBF) in transcription in the context of chromatin-assembled DNA, we used regularly spaced nucleosomal DNA using topoisomerase IIα (topo IIα) and α2(1) collagen promoter templates, which were subsequently reconstituted in an in vitro transcription reaction. Binding of CBF to the nucleosomal wild-type topo IIα promoter containing four CBF-binding sites disrupted the regular nucleosomal structure not only in the promoter region containing the CBF-binding sites but also in the downstream region over the transcription start site. In contrast, no nucleosome disruption was observed in a mutant topo IIα promoter containing mutations in all CBF-binding sites. Interestingly, CBF also activated transcription from nucleosomal wild-type topo IIα promoter. In this experiment, a promoter containing one wild-type CBF-binding site was activated very weakly, whereas the promoter containing mutations in all sites was not activated by CBF. A truncated CBF that lacked the glutamine-rich domains did not activate transcription from nucleosomal wild-type topo IIα promoter but disrupted the nucleosomal structure about as much as did the binding of full-length CBF. Two nucleosomal mouse α2(1) collagen promoter DNAs, one containing a single and the other containing four CBF-binding sites, were also reconstituted in an in vitro transcription reaction. None of the nucleosomal collagen promoters was activated by CBF. However, both of these collagen promoters were activated by CBF when the transcription reaction was performed using naked DNA templates. Binding of CBF to the nucleosomal collagen promoter containing four binding sites disrupted the nucleosomal structure, similarly as observed in the topo IIα promoter. Altogether this study indicates that CBF-mediated nucleosomal disruption occurred independently of transcription activation. It also suggests that specific promoter structure may play a role in the CBF-mediated transcription activation of nucleosomal topo IIα promoter template.

The chromatin structure plays an important role in the regulation of transcription in eukaryotic cells. Generally the chromatin produces a repressive environment for transcription and the repression could occur at multiple steps of transcription activation (1, 2). Studies from many laboratories showed that specific DNA-binding proteins that bind to a particular promoter result in nucleosomal disruption or remodeling surrounding the promoter region, which then allows recruitment of coactivators and general transcription machinery to activate transcription (3–5). Mammalian promoters usually contain binding sites for various specific DNA-binding proteins. It is now recognized that the binding of multiple proteins may be required to disrupt the nucleosomal structure surrounding the promoter in a way that presets the promoter for subsequent transcription activation (6–8).

The mammalian CCAAT-binding transcription factor CBF/NF-Y is a heterotrimeric protein consisting of CBF-A, CBF-B, and CBF-C (9, 10). CBF-A and CBF-C contain histone-fold motifs that are similar to histones H2B and H2A, respectively (11, 12). CBF contains two glutamine-rich transcription activation domains, one present in CBF-B and the other in CBF-C (13). A survey of mammalian promoters showed that about 25% contain CBF-binding sites (14). The CBF subunits are ubiquitously expressed in all mammalian tissues.

To understand the in vivo function of CBF, we have recently inactivated CBF in mouse fibroblast cells by expressing a dominant-negative CBF-B mutant that inhibits DNA binding of CBF protein (15). This study showed that the mutant but not the wild-type CBF-B retarded fibroblast cell growth. Analysis of the mRNA profile showed that the inactivation of CBF in fibroblasts decreased the expression of only a small number of cellular genes, many of which are regulated during cell growth. This study suggested that in cultured mammalian cells CBF may primarily be involved in transcription of growth-regulated genes. This is also further supported by the observation that the promoters of various growth-regulated genes such as thymidine kinase (16), E2F1 (17), topoisomerase IIα (topo IIα) (18, 19), cyclin B1 (20), and CDC25C (21) contain multiple CBF-binding sites. Altogether this analysis implied that the in vivo transcription activity of only a subset of all promoters containing CBF-binding sites was affected by the inhibition of CBF binding in fibroblasts.

In this regard it is important to mention that promoters of many tissue-specific genes that are not expressed in fibroblasts

* This work was supported by National Institutes of Health Grant R01 AR46824 (to S. N. M.) and NC1 Grant CA49515 (to B. d. C.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: CBF, CCAAT-binding factor; topo, topoisomerase; bp, base pair; PCR, polymerase chain reaction; Mnase, micrococcal nuclease.

This paper is available on line at http://www.jbc.org
also contain CBF-binding sites. So it is conceivable that the in vivo function of CBF is dependent on the state of chromatin structure in the promoter. Indeed recent studies of the chromatin structure of the Xenopus hsp70 promoter showed that the two CBF-binding sites in this promoter were required to generate a nuclease-hypersensitive site in chromatin and played an important role in acetylation-responsive transcription in Xenopus oocyte nuclei (22). This implied that CBF binding to this promoter altered the local chromatin architecture in the promoter so that recruitment of other transcription factors was facilitated. However, it remained to be determined whether such a function of CBF is specific to the hsp70 promoter or generally applicable to other promoters.

It is possible that transcription of growth-regulated genes whose promoters contain multiple CBF-binding sites are highly dependent on CBF. The promoter of the mouse topo IIα gene has been recently isolated (18). The most striking feature of this promoter is that it contains seven CBF-binding sites within the 210-bp upstream sequence of this gene and lacks a consensus TATA motif. The topo IIα is one of the two isoforms of DNA topoisomerase II enzyme which is a ubiquitous nuclear enzyme that can alter the topology of DNA and plays an important role during many cellular processes such as transcription, replication, recombination, and chromosome segregation.

The expression of topo IIα is regulated during cell growth, with an activity that peaks at G2/M phase of the cell cycle; in contrast expression of the other isoform, topo IIβ, is unchanged during the cell cycle. Thus it is believed that the topo IIα isoform plays a major role during chromosome segregation in mitosis (18).

Here we analyzed the role of CBF in transcription of topo IIα promoters using in vitro reconstituted nucleosomal assembled DNA templates. We found that binding of CBF to the wild-type promoter disrupted regular nucleosomal structure not only at the CBF binding regions but also in the downstream promoter region. Interestingly, CBF also strongly activated transcription of nucleosomal topo IIα promoter even after nucleosomes were reconstituted with histone H1, which strongly repressed transcription. Altogether our results showed that transcription of nucleosomal topo IIα promoter was highly dependent on CBF-binding sites. Furthermore, we showed that although CBF activated transcription of collagen promoters in naked DNA template, it did not activate transcription of nucleosomal collagen promoter template. This study suggested that a promoter-specific mechanism plays a role in the CBF-dependent transcription activation of nucleosomal topo IIα template.

MATERIALS AND METHODS

Plasmid Construction—The reporter plasmids FC1 and FC2 contained the sequence of the mouse α2(I) collagen promoter from pc1 and pFC2 (23). These promoter sequences were amplified by PCR and inserted between the KpnI and Xhol sites in the pgL3-basic vector (Promega). The reporter plasmid PGL3-350 was constructed by inserting the sequence of the mouse α2(I) collagen promoter, amplified by PCR from pH 6 (24), between the Xhol and HindIII sites in the pgL3-basic vector. To construct the 4CCAAT reporter plasmid, the sequence between −170 and +110 of the mouse topoisomerase IIα gene promoter (18) was amplified by PCR and was cloned between the Saci and Xhol sites in the pgL3-basic vector. The reporter templates M1–4, W1, and W3, carrying mutations of the CBF-binding sites in the topo IIα promoter, were obtained by introducing a point mutation (CCAAT to CCAAA) within the 210-bp upstream sequence of this gene and lacks a CBF binding site. The reporter templates M1–4, W1, and W3, carrying mutations of the CBF-binding sites in the pGL3-basic vector.

Generation of Recombinant Proteins—Full-length and truncated CBF proteins were expressed in Escherichia coli as fusion proteins with glutathione S-transferase as described previously (13, 23).

Nuclear Extracts—HeLa cells were grown in suspension in Joklik medium supplemented with 5% calf serum at the density of 0.6 × 10^6 cells/ml. Nuclear extracts were prepared as described (26) and then were depleted of CBF as described previously (23) by mixing with a DNA affinity resin in which the DNA sequence of the mouse α2(I) collagen gene promoter from −105 to −64 was covalently linked to Sepharose.

Chromatin Assembly—The S190 extracts were prepared from 0- to 6-h Drosophila embryos (from Canton-S wild-type flies generously provided by W. Mattoo, M. D. Anderson Cancer Center, Houston, TX) as described by Kadonaga and co-workers (27, 28). These extracts were supplemented with exogenous core histones prepared from HeLa cells. HeLa cells were grown for 24 h at 37 °C in medium containing 8 mM sodium butyrate and then used for the preparation of chromatin-containing histones according to Wolff and Hayes (29). The histones were fractionated according to the procedure of Simon and Felsenfeld (30). In a typical assembly reaction, 30 μl of S190 extract was incubated with 1.6 μg of purified core histones in RO buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 0.5 mM EGTA, 10 mM MgCl2, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) at room temperature for 30 min. To this sample, 10× ATP mix (300 mM creatine phosphate, 30 mM ATP, 1 μg of creatine phosphokinase, 26 mM MgCl2) was added and incubated for 5 min on ice. One microgram of plasmid DNA (preincubated or not with CBF for 20 min on ice) was then added, and chromatin assembly was allowed to proceed for 4.5 h at 27 °C. Purified histones H1, H4, and 0.8 μg (kindly provided by S. Majumder, M. D. Anderson, Houston, TX), were added in some reactions.

In Vitro Transcription—The topo IIα promoter constructs and the α2(I) collagen promoter constructs, either as naked plasmid DNA or as chromatin-reconstituted DNA (taken as an aliquot of the chromatin assembly reaction mixture), were incubated with the transcription mix (1% polyvinyl alcohol, 1% polyethylene glycol, 1 mM NTPs, 5 mM MgCl2 final concentration), HeLa nuclear extract, and p120 plasmid DNA (23). The templates were transcribed for 1 h at 27 °C. Transcription reactions were stopped with transcription stop buffer (0.4 M NaOAc, pH 5.2, 1% sodium chloride, 1% phenol/chloroform extraction, and ethanol precipitation with glyogen as carrier. RNAs were analyzed by primer extension as described previously (23).

Micrococcal Nuclease and DNase Digestion—The detergent was digested immediately after chromatin assembly with micrococcal nuclease (Mnase) for 5, 10, and 20 min or with DNase I for 10 min. The digestion was stopped by adding stop buffer (TE, EDTA, RNase I) for 15 min on ice. This was followed by proteinase K treatment. The DNA was purified by phenol/chloroform extraction, chloroform extraction, and ammonium acetate/ethanol precipitation with glyogen as carrier.

For Mnase ladder, DNA was subjected to electrophoresis on a 1.5% agarose gel and in some cases transferred to a nylon membrane for Southern blot analysis. After prehybridization in hybridization buffer (6.66% SDS, 0.33 M NaPO4, pH 7.2), the membrane was hybridized with labeled oligonucleotides at 45 °C for 1 h. Washes were carried out in wash buffer (2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS) briefly at room temperature, 15-min wash in 2× SSC, 0.1% SDS at 45 °C, and 15-min wash in 1× SSC, 0.1% SDS at 45 °C. Sequences of the various oligonucleotides used are as follows: plasmid-680 (5′-GGTACCGCTTCGACCCGCGA-3′); plasmid-680 (5′-TCTTATCAGTGACTGCAGTC-3′); prom-46 (5′-TTCCCGCTTCTGCACTGCAGTATCTG-3′); prom-37 (5′-CTACCGACCTTCTCCTAACCG-3′); prom-109 (5′-AACCGCGCTGCAGGCTTGACGCGAGGCGA-3′); plasmid+186 (5′-CTACCGACCTTCTCCTAACCG-3′); prom-207 (5′-TCTTATCAGTGACTGCAGTC-3′); plasmid+283 (5′-GGTACCGCTTCGACCCGCGA-3′).

Plasmid Footprinting—DNA, digested with either Mnase or DNase I, was subjected to linearized expression vector. The linearized expression vector was mixed by adding in a hot-start PCR tube the reaction buffer with the labeled oligonucleotide and dNTPs. The PCR tube was heated at 90 °C for 30 s and cooled to room temperature to form the DNA-DNA duplex. The second reaction containing the DNA, the reaction buffer, and Vent (exo ) polymerase (New England Biolabs) was added to the PCR tube. The extension program was run in a PCR machine (4 min at 95 °C, 8
cycles of 1 min at 94 °C, 3 min of annealing at 45 °C for primer plasmid +283 and primer plasmid +186, 30 s at 74 °C, and hold at 18 °C). After NaOAc/EtOH precipitation, the DNA were analyzed on a 6% sequencing gel using a NaOAc gradient (top buffer, 0.5× TBE; bottom buffer, 1× TBE, 1 m NaOAc).

RESULTS

The Activity of Topoisomerase IIα Promoter Is Highly Dependent on CBF-binding Sites in Fibroblast Cells—To analyze the role of CBF-binding sites in topo IIα promoter activity, we inserted the sequence −251 to +110 of the topo IIα gene in a vector 5’ to a luciferase reporter gene. Transfection of this construct into mouse fibroblast cells showed a high level of promoter activity (data not shown). Deletion of the promoter sequence from −170 to +110, which contains four CBF-binding sites, decreased promoter activity only about 20%. However, further deletion significantly reduced promoter activity. We used the −170 to +110 construct, which is designated here as 4CCAAT, as a wild-type background to introduce mutations into the CBF-binding sites. Each of the CBF-binding sites in this construct was mutated by single nucleotide substitution mutations from CCAAT to CCAAA or ATGG to TTTGG (Fig. 1A). A promoter construct containing mutations in all four CBF-binding sites (M1–4) showed a 50-fold decrease in promoter activity. The promoter containing a single CBF-binding site (W1 or W3) retained significant promoter activity but was 3–4-fold lower than that of the 4CCAAT construct (Fig. 1B). This result indicated that the activity of the topo IIα promoter is highly dependent on CBF-binding sites in vivo. In this regard it is important to note that not all promoters containing a CBF-binding site are dependent to the same extent on their CBF-binding site. For example, we showed previously that a mutation in the single CBF-binding site of the mouse α2(1) collagen promoter resulted in only a 4-fold decrease of promoter activity (24). To test the possibility that the dependence of promoter activity on CBF-binding sites may increase with the number of CBF sites in the promoter, we constructed a promoter that contains four CBF-binding sites placed before the minimal promoter of the α2(1) collagen gene, named here as FC1. A mutant promoter, FC2, in which the four CBF-binding sites of FC1 were mutated by single nucleotide substitution, was also constructed (Fig. 1A). Previously, using an in vitro reconstituted transcription system, we showed that CBF strongly activated the transcription of the FC1 but not the FC2 promoter (23). In DNA transfection experiments the promoter activity of FC1 was only 4-fold higher than FC2, similar to the promoter (23). In DNA transfection experiments the promoter activity of FC1 was only 4-fold higher than FC2, similar to the overall promoter activity of FC1 was about 8–10-fold lower than the 4CCAAT promoter of topo IIα (Fig. 1B). This result suggests that the presence of multiple CBF-binding sites in the promoter does not always contribute higher dependence of promoter activity to the CBF sites.

Binding of CBF to Nucleosomal DNA Strongly Disrupts the Regular Nucleosomal Structure—Recent studies showed that the two CBF-binding sites in the Xenopus hsp70 promoter play a role that presets the chromatin structure, a process that is required for transcriptional activation by heat shock (22). This suggests that the CBF-dependent transcription activation in mammalian cells may be due, at least in part, to CBF-mediated nucleosome disruption, which may in turn facilitate recruitment of general transcription factors to form a preinitiation complex.

To test this possibility, we studied the interaction of recombinant CBF with nucleosomal promoter DNA reconstituted in vitro. The DNA templates containing wild-type and mutant topo IIα promoters were incubated with Drosophila embryo extracts and purified human core histones and an ATP-regenerating system as described by Kamakaka et al. (28). Formation of nucleosomes in the DNA template was verified by Mnase digestion analysis. This showed formation of DNA ladders of 146 bp and multiples of 146 bp, thus indicating that regularly spaced nucleosomal arrays had formed on the DNA template similar to that found in vivo in mammalian or other eukaryotic cells (Fig. 2B, upper panel, lanes 1–3 and 10–12). Addition of CBF to the incubation mixture before (lanes 7–9 and 16–18) or after formation of nucleosomes (lanes 4–6 and 13–15) did not change the overall nucleosome pattern of the DNA template. To monitor specifically the nucleosome structure over the promoter sequence, we performed a Southern blot analysis, hybridizing the Mnase-digested DNA with a labeled oligonucleotide probe corresponding to the +37-bp region in the
This showed that all DNA fragments generated with wild-type nucleosomal DNA, formed without CBF, hybridized strongly with the oligonucleotide probe (Fig. 2B, lower panel, lanes 1–3). This indicated that the regular nucleosomal structure was present over the promoter region probed with the oligonucleotide. In contrast, incubation of CBF with the nucleosomal wild-type DNA significantly reduced hybridization (lanes 4–6), and more strikingly, preincubation of CBF before formation of the nucleosomes resulted in an almost complete loss of hybridization (lanes 7–9) of the oligonucleotide with the DNA ladders. Incubation of CBF with nucleosomal mutant DNA, however, did not cause any change in hybridization efficiency of the oligonucleotides with the DNA ladders (lanes 10–18). Recently it was reported (31) that CBF-A/CBF-C interacts with the histone H3-H4 complex in vitro. However, incubation of CBF-A/CBF-C or CBF-B alone with the topo IIα promoter (see Fig. 2A). 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wild-type DNA before or after nucleosome formation did not result in any alteration in hybridization efficiency of the oligonucleotides with the DNA ladders (data not shown). This result indicated that specific binding of CBF to the CCAAT motifs in the topo II promoter strongly disrupted the regular nucleosomal structure over the nucleosomal promoter region.

To monitor the extent of disruption, we performed a Southern blot analysis of Mnase-digested nucleosomal wild-type and mutant DNA, which was formed after preincubation with CBF, with a series of oligonucleotides corresponding to different regions of the promoter and vector DNA (see Fig. 2A). Hybridization of each oligonucleotide with Mnase-digested wild-type or mutant nucleosomal DNA generated without CBF showed patterns of hybridization to DNA ladders that were identical to those observed in Fig. 2B, lower panel, lanes 1–3 and 10–12 (data not shown). All oligonucleotides also hybridized almost equally with the DNA ladders of mutant nucleosomal DNA preincubated with CBF (Fig. 2C, lower panel, lanes 1–18). In contrast, hybridization of the +109 and +207 oligonucleotides to the DNA ladder of wild-type nucleosomal DNA preincubated with CBF was very significantly reduced, whereas hybridization of −66 and +283 oligonucleotides was reduced modestly. The same DNA ladders, however, hybridized very efficiently with the −680 and −167 oligonucleotides. This indicated that binding of CBF to the nucleosomal topo II promoter disrupted the regular nucleosomal structure over the promoter region from −66 to +207.

To monitor more precisely the extent of CBF-mediated nucleosomal disruption, we analyzed the Mnase-digested nucleosomal DNA with the primer extension method using a radiolabeled primer that hybridized at a downstream promoter region (Fig. 3A). Since Mnase cleaves DNA outside of and not within the nucleosome, the primer extension bands should correspond to various nucleosomal positions over the promoter DNA. Our comparison of primer extension bands of wild-type nucleosomal DNA generated with or without CBF showed that binding of CBF protected promoter DNA and augmented Mnase cleavage (lanes 1 and 2). The protected Mnase cleavage regions were located over the CBF-binding sites from positions −66 to −117, whereas regions of augmented cleavage are located both upstream and downstream of CBF-binding sites. Most notably, binding of CBF strongly increased Mnase cleavage at the region containing the start of transcription at +1, consistent with the observation in Fig. 2C. In contrast, incubation of CBF with nucleosomal promoter DNA containing mutations in all four CBF sites (M1–4) did not result in either protection or augmentation of Mnase cleavage in the promoter region (lanes 5 and 6). However, incubation of CBF with nucleosomal promoter DNA containing a single wild-type CBF site (W1) located at position −26 resulted in only a small increase of Mnase cleavage over the promoter region at the start of transcription (lanes 3 and 4), indicating that the multiple CBF-binding sites are required for strong disruption of nucleosomal structure over the start of the transcription region. Overall, the results of Fig. 3A indicate that CBF specifically disrupted the nucleosomal structure over the topo II promoter region from approximately −200 to +46 bp. However, it was not clear from this experiment whether this nucleosomal disruption was due to direct interactions of CBF with the four CBF sites in topo IIα promoter within the nucleosome.

To determine the sites of interaction of CBF in nucleosomal DNA, we used DNase I cleavage followed by primer extension analysis. Since DNase I cleaves DNA within the nucleosome, this method allows one to envision the interaction of CBF with DNA within the nucleosome. Nucleosomes were formed with the wild-type topo IIα promoter 4CCAAT construct in the presence or absence of CBF, as in Fig. 3A. Nucleosomal DNA was first cleaved by DNase I and then the digested DNA was visualized by primer extension analysis using the labeled primer as in the earlier experiment (Fig. 3B, lanes 1–3). In lane 2 DNA was preincubated with DNA before formation of nucleosomes, and in lane 3 CBF was incubated after formation of nucleosomes. The primer extension analysis shows that the preincubation of CBF with nucleosomal DNA resulted in both protection and formation of characteristic hypersensitivity sites of DNase I cleavage in all four CBF-binding regions. Similar hypersensitive and protected sites were also observed when CBF was incubated after formation of nucleosomes. This indicates that all four CBF-binding sites of the topo IIα promoter interacted with CBF in nucleosomal templates and that CBF also bound to the CBF-binding sites in the promoter even after formation of nucleosomes.

**CBF Activates Transcription of Topoisomerase IIα Promoter within the Nucleosome—**To determine whether CBF activates transcription of the topo IIα promoter in chromatin, we reconstituted nucleosomal DNA with recombinant CBF in an in vitro...
transcription reaction using HeLa cell nuclear extracts. Transcription of wild-type topo IIα promoter in a naked DNA template showed that this promoter contained three major transcription start sites, designated I–III (Fig. 4A, lane 1). Addition of α-amanitin inhibited transcription from all three start sites, indicating that the three start sites in the promoter were transcribed by RNA polymerase II (data not shown). When endogenous CBF was partially depleted from nuclear extracts by incubating with a DNA affinity resin containing a CBF-binding site, transcription of topo IIα promoter from start site II was specifically decreased (lane 2). Interestingly, addition of CBF to the depleted extract activated transcription of topo IIα promoter from start site II very strongly and modestly from start site III; in contrast, transcription from this start site I was decreased upon addition of CBF. In the reaction mixture containing mutant topo IIα promoter (M1–4) with mutations in all the four CBF sites, there was essentially no activation of transcription when CBF was added (lanes 8–10). It is important to note that in the mutant promoter there was much less transcription from start site II than from start site I (lane 8), consistent with the finding that transcription from start site II was dependent on CBF-binding sites. Altogether, these results indicate that CBF specifically activated transcription of the topo IIα promoter.

When transcription was performed using a wild-type nucleosomal topo IIα promoter template, transcription from all three start sites was observed (lane 4). Although the presence of nucleosomes in the promoter usually strongly represses transcription, surprisingly the topo IIα promoter within chromatin was transcribed efficiently and was only modestly repressed when compared with transcription of naked DNA. With partially CBF-depleted nuclear extract, transcription from start site II was decreased (lane 5). When CBF was incubated with the promoter DNA either before or after formation of nucleosomes and then reconstituted in the transcription reaction, strong activation of transcription was seen from start site II and modest activation from start site III (lanes 6 and 7), similar to what was observed in the naked DNA template. In contrast, when transcription was performed using mutant nucleosomal promoter DNA (M1–4), almost no transcription from any start site of the promoter was observed (lanes 11 and 12). Addition of CBF resulted in very low level activation from start site II (lanes 13 and 14).

A mutant promoter construct containing a single wild-type CBF site (W1) was also transcribed in vitro both as a naked DNA template and nucleosomal template. As shown in Fig. 3A, binding of CBF to W1 did not cause much nucleosomal disruption over the start of transcription region. In a naked DNA template CBF activated transcription of W1 promoter (Fig. 4B, lanes 1 and 2); however, the level of activation was five times lower than that of the wild-type template containing four CBF sites. Transcription of the W1 nucleosomal template resulted in almost no transcription from any of the three start sites of the promoter, but addition of CBF resulted in a significant transcription activation, specifically from start sites II and III (lanes 3 and 4). This indicates that CBF activated transcription of the nucleosomal topo IIα promoter containing a single CBF-binding site, even though binding of CBF to this promoter...
resulted in only a modest nucleosomal disruption. Altogether this result indicated that CBF specifically activates transcription of nucleosomal topo IIα promoter. The strong transcriptional repression from all three start sites of the mutant nucleosomal promoters suggests that the multiple CBF-binding sites in the topo IIα promoter play an important role to support basal transcription from all three start sites in chromatin.

Since the activity of the nucleosomal topo IIα promoter was not completely repressed without the addition of recombinant CBF, it was not clear whether CBF-dependent activation of the nucleosomal promoter required a partially derepressed state of nucleosomes. Thus to test whether CBF could activate transcription when the promoter is in a completely repressed state, we performed the nucleosome reconstitution in the presence of histone H1, which is known to repress transcription (32). Mnase digestion of nucleosomal DNA generated with histone H1, which is known to repress transcription (32), showed that binding of CBFmut to the nucleosomal promoter either with a naked DNA template or with a nucleosomal template (Fig. 4D, lanes 1–5). DNase I cleavage analysis showed that CBFmut bound to the nucleosomal promoter as well as full-length CBF (Fig. 5, lanes 1–6). Interestingly, Mnase cleavage analysis showed that binding of CBFmut resulted in a strong nucleosomal disruption, much like the nucleosomal disruption produced by full-length CBF (lanes 1–3). This result indicates that the activation domains of CBF did not play a role in the CBF-mediated nucleosomal disruption, although these domains mediated transcriptional activation of the nucleosomal topo IIα promoter.

CBF Disrupts Nucleosomal Structure but Does Not Activate Transcription of a Nucleosomal Collagen Promoter—CBF-binding sites are present in promoters of many mammalian genes, including genes that are expressed in specific tissues or cell types. The CBF protein is expressed ubiquitously in all mammalian cell types. However, transcription of tissue-specific promoters containing CBF-binding sites is restricted to specific
cell types. This suggests that although CBF activates transcription of the topo IIα promoter in chromatin, it may not activate similarly all mammalian promoters containing CBF-binding sites. To test this possibility, we reconstituted the in vitro transcription reaction with the promoter of the mouse α2(I) collagen gene. The collagen promoter contains a single CBF-binding site located at −80 within the pGL3-350 construct (Fig. 6A). We also constructed a promoter, FC1, containing four tandem collagen CBF-binding sites upstream of a minimal collagen promoter (−40 to +54). As expected from our previous studies (13, 23), both promoters were activated by CBF in the transcription reactions containing naked DNA (Fig. 6B, lanes 1–3 and 8–10). When the transcription reaction was performed with nucleosomal promoters, almost no transcription from either collagen promoter was observed (lanes 4, 5, 11, and 12), indicating that the presence of nucleosomes strongly repressed the transcription activity of these promoters. Surprisingly, addition of CBF to nucleosomal promoter did not activate the collagen promoters (lanes 6 and 7, and 13 and 14). This result indicated that CBF is unable to activate transcription of a collagen promoter even if four CBF-binding sites are introduced in a configuration similar to the naturally occurring topo IIα promoter.

To examine better the CBF-mediated nucleosome disruption of the collagen promoters, we digested the nucleosomes with Mnase and then analyzed them by primer extension. Incubation of CBF with the nucleosomal FC1 collagen promoter containing four CBF-binding sites resulted in both protection and augmentation of Mnase cleavage (Fig. 7A, lanes 1–3). Although the alteration of Mnase cleavage was also observed downstream of the CBF-binding region, between CBF site I and the TATA box (lanes 2 and 3). In contrast, incubation of CBF with the nucleosomal pGL3-350 collagen promoter containing a single CBF-binding site did not significantly change the Mnase cleavage pattern (lanes 5 and 6). Thus CBF strongly disrupted nucleosomal structure in the collagen promoter containing four CBF sites but not in the promoter containing a single CBF site. The interaction of CBF with the nucleosomal collagen promoter was revealed by Dnase I cleavage followed by primer extension analysis (Fig. 7B). This showed that incubation of CBF with the FC1 collagen construct before or after formation of nucleosome resulted in formation of characteristic hypersensitive Dnase I cleavage and protected sites over all four CBF sites (lanes 2 and 3). Similarly incubation of CBF with the pGL3-350 construct also resulted in formation of hypersensitive Dnase I cleavage sites over the single CBF-binding site in the promoter (lanes 5 and 6). This result indicated that CBF interacts with the nucleosomal collagen promoters much as it does with the nucleosomal topo IIα promoter. Altogether this analysis is consistent with the earlier observation that binding of multiple CBF molecules to the collagen promoter is required for CBF-mediated nucleosomal disruption.

Comparison of CBF-mediated nucleosomal disruption in 4CCAAT topo IIα and FC1 collagen promoters showed that although the overall nucleosomal disruption in these two promoters is similar, a difference in disruption was seen in the region around the start of transcription. In the topo IIα promoter, the start site of transcription activated by CBF was located within the disrupted nucleosomal region, whereas in the FC1 collagen promoter almost no nucleosomal disruption...
was observed over the area of the start of transcription (Fig. 8). This result may account for the inability of CBF to activate transcription of the FC1 collagen promoter in chromatin.

### DISCUSSION

We showed that the in vivo transcription activity of the topo IIα promoter was highly dependent on the presence of several CBF-binding sites, since the activity of this promoter was 50 times higher than the activity of a mutant promoter in which four CBF-binding sites were mutated. In contrast, the transcription activity of a collagen promoter with the same number of CBF sites as the topo IIα promoter was only four to five times higher than that of a mutant collagen promoter in which these sites were mutated. Thus this collagen promoter is much less dependent on CBF-binding sites than the topo IIα promoter. This suggests that the in vivo CBF-mediated transcription activation is dependent on promoter context. To test whether the difference in CBF response might be accounted for by the presence of nucleosomes, we analyzed the role of CBF with in vitro reconstituted nucleosomal promoter DNAs.

Our results showed that binding of multiple CBF molecules to a promoter caused extensive nucleosomal disruption, whereas a weak disruption was observed in the promoter containing a single CBF-binding site. We hypothesize that in vivo CBF binds to many promoters with CBF-binding sites, but binding of CBF by itself will disrupt the regular nucleosomal structure only in promoters that contain multiple CBF-binding sites. In a promoter with a single site, we speculate that other transcription factors could be instrumental together with CBF in causing chromatin disruption.

In our study recombinant CBF specifically activated transcription of the nucleosomal topo IIα promoter. Although the topo IIα promoter contained three major transcription start sites, CBF activated transcription mainly from one of them, suggesting that another DNA element might be required for CBF-mediated transcription activation from this single start site. Interestingly, mutations in all four CBF-binding sites resulted in no transcription from any of the three start sites in the nucleosomal mutant template. Similarly, almost no transcription was seen from the nucleosomal promoter containing a single CBF-binding site (W1). This indicated that nucleosomes repressed transcription of the wild-type promoter only modestly but completely repressed transcription of the mutant promoters. We interpreted this result as indicating that multiple CBF-binding sites in the wild-type promoter, in the presence of CBF in nuclear extracts, mediates nucleosomal disruption and allows transcription from all the start sites of the wild-type promoter even though transcription from two of the start sites is not stimulated by CBF. Transcription of the wild-type promoter was repressed almost completely when nucleosomes were reconstituted in the presence of histone H1, a situation that has been observed in other promoters. In this highly repressed condition, CBF could activate transcription of the wild-type promoter, indicating that CBF is able to overcome this chromatin-mediated repression.

CBF-mediated transcription activation of the nucleosomal topo IIα promoter requires activation domains of CBF. However, truncated CBF without activation domains binds to the nucleosomal promoter and disrupts nucleosomal structure similarly to full-length CBF. It is possible that the histone-fold motifs of CBF-A and CBF-C, which are required for formation of CBF-DNA complex, may also play a role in CBF-mediated nucleosomal disruption. Although recent studies of Caretti et al. (31) showed an interaction between CBF-A/CBF-C and histones H3/H4 in the absence of CBF-binding sites, we did not observe any modulation of the nucleosome structure in the wild-type topo IIα promoter in the presence of CBF-A/CBF-C only (data not shown). This indicates that the CBF-A/CBF-C heterodimer by itself does not disrupt the nucleosomal structure. Thus if the histone fold motifs of the CBF-A/CBF-C dimer play a role in the nucleosomal disruption, it may function within the CBF-DNA complex. The activation domains of CBF do not play any role in CBF-mediated nucleosomal disruption, thus suggesting that the activation function of CBF is distinct from the nucleosomal disruption function. In this respect CBF may be similar to several other transcription factors that activate transcription and disrupt nucleosomes by separate mechanisms (32–34). Altogether these results lead us to speculate that in vivo CBF can access the topo IIα promoter within chromatin and interacts with multiple binding sites in this promoter. Solely through these interactions, CBF disrupts the local nucleosomal structure so as to allow transcription from this promoter to occur. However, in addition to disrupting the nucleosomal structure, CBF by itself also contributes to activation of transcription, along with other transcription factors that bind to this promoter.

Although binding of CBF to a nucleosomal collagen promoter disrupted the nucleosomal structure, transcription activation of the promoter did not occur. This result was in stark contrast to the CBF-dependent chromatin disruption of the nucleosomal
topo IIα promoter that resulted in strong activation of this promoter. It strongly suggests that CBF-mediated nucleosomal disruption is totally separated from CBF-mediated transcription activation. Two possible mechanisms might account for CBF-mediated transcription activation of a nucleosomal promoter. One of the differences between topo IIα and collagen promoters is the basal promoter sequences. The collagen promoter contains a consensus TATA motif, and the transcription is initiated from a single start site. The topo IIα promoter does not contain a TATA motif, and transcription is initiated at multiple start sites, a process that is probably mediated by various initiator elements. Thus the mechanism by which the basal preinitiation transcription complexes are recruited to these two promoters is different. A first possible mechanism is that CBF might facilitate recruitment of a basal transcription complex to the nucleosomal promoter in the context of an initiator element, but not in the context of a TATA element, and that CBF would favor activation of the topo IIα promoter through an initiator element. A second hypothesis is based on the extent of CBF-mediated nucleosomal disruption in the downstream promoter regions of the topo IIα and collagen promoters (Fig. 8). One of the major rate-limiting steps in the transcription activation process of a nucleosomal promoter is the recruitment of basal transcription complexes. Since CBF binding to the topo IIα promoter containing either four or single wild-type collagen sites resulted in nucleosomal disruption that extended over the start site of transcription and activation of transcription from this site, it is likely that binding of the basal transcription complex to this start site is highly favored as a result of this nucleosomal disruption. In contrast the CBF-mediated nucleosomal disruption in the collagen promoter does not include the TATA element and the transcription start site, thus suggesting that recruitment of the basal transcription complex might be inhibited due to the absence of nucleosomal disruption over the TATA motif. Altogether this analysis suggests that the structure of a promoter may determine whether CBF will function as a transcription activator in chromatin.

In this regard one of the well studied examples is the role of CBF in transcription activation of the major histocompatibility complex class II promoter (35). The activity of this promoter is determined by the arrangement of the CBF-binding site (Y element) with an X box element that interacts with RXP proteins. Changing the orientation of these two elements resulted in an almost complete inactivation of the promoter. Interestingly, recent studies showed that a specific arrangement of these two elements is required for the recruitment of a specific transcription coactivator, CIITA, which in turn leads to activation of transcription of this promoter (36). Thus it is possible that other specific elements in the topo IIα promoter similarly favor CBF-dependent transcription activation in chromatin.

Acknowledgments—We are grateful to William Mattox, Elaine Mcguillin, and Brigitte Daualder for their help in setting up the Drosophila mass cultures. We acknowledge Jessica Tyler in Jim Kadonaga’s laboratory for providing the protocol for linear amplification. We thank Walter J. Pagel for editorial assistance. The DNA Core Sequencing Facility was the recipient of NCI Grant CA16672 from the National Institutes of Health.

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CBF/NF-Y Functions Both in Nucleosomal Disruption and Transcription Activation of the Chromatin-assembled Topoisomerase II α Promoter: TRANSCRIPTION ACTIVATION BY CBF/NF-Y IN CHROMATIN IS DEPENDENT ON THE PROMOTER STRUCTURE

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J. Biol. Chem. 2001, 276:40621-40630.
doi: 10.1074/jbc.M106918200 originally published online August 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106918200

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