CCAAT Binding Factor (CBF) Binding Mediates Cell Cycle Activation of Topoisomerase IIα

CONVENTIONAL CBF ACTIVATION DOMAINS ARE NOT REQUIRED*

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Qianghua Hu‡, Chitrakala Bhattacharyya‡, and Sankar N. Maity§
From the Department of Molecular Genetics, The University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030

To understand the role of the CCAAT binding factor (CBF) in transcription during the cell cycle, we studied the mouse topoisomerase IIα (topo IIα) promoter, which is activated during the late S and G2/M phases of the cell cycle and contains multiple CBF binding sites. Mutational analysis of the promoter shows that CBF binding to an inverted orientation of the CCAAT motif in the topo IIα promoter, but not to a direct orientation, is required for transcription activation during the cell cycle. In contrast, analysis of the promoter in an in vitro reconstituted transcription system shows that CBF activates transcription of the topo IIα promoter irrespective of the orientation of the CBF binding sites. This analysis demonstrates that only one of the three transcription start sites of the topo IIα promoter is stimulated by CBF, indicating that transcription activation by CBF is dependent on basal promoter structure. Interestingly, mutations of the start site that abolish CBF-dependent transcription activation in vitro do not inhibit activation of the promoter during the cell cycle. Consistent with this observation, expression of a truncated CBF-B subunit lacking a transcription activation domain, which inhibits activity of a collagen promoter, does not affect activity of the topo IIα promoter in fibroblast cells. In contrast, expression of an allele-specific CBF-B mutant that binds high affinity to a mutant CBF binding site containing a CCAAC motif revives transcription activation of an inactive mutant topo IIα promoter containing CCAAC during the cell cycle. Altogether, this study indicates that CBF binding, but not conventional CBF activation domains, are required for activation of the topo IIα promoter during the cell cycle. Considering these results together with results of another recent study, we hypothesize that binding of CBF that disrupts the nucleosomal structure in the topo IIα promoter is a major function of CBF by which it regulates the cell cycle-dependent transcription of the topo IIα promoter and possibly many other cell cycle-regulated promoters containing CBF binding sites.

DNA topoisomerase II (topo II)† is a ubiquitous nuclear enzyme that is involved in various cellular functions, such as replication, chromatin condensation, and segregation of newly synthesized chromatin pairs. Mammalian cells contain two topo II isoforms, 170-kDa topo IIα and 180-kDa topo IIβ. Although the two isoforms are structurally very similar, they differ with respect to several biochemical and pharmacological properties, including sensitivity to topo II-targeting drugs and regulation of synthesis during cell cycle. Whereas the level of topo IIβ is constant throughout the cell cycle, the expression of topo IIα varies during the cell cycle. The topo IIα level is very low at G0/G1 phase, begins to increase in late S phase, and is high at G2/M phase. Moreover, the expression of the topo IIα gene is almost undetectable in quiescent or differentiated cells but is present in proliferating cells of all normal tissues of mice and humans as well as in various human tumors. Thus, it is believed that the topo IIα isoform plays a major role during chromosome segregation in proliferating cells (1, 2).

To understand transcriptional regulation of the topo IIα gene during cell proliferation, the promoter of the topo IIα gene was isolated from various mammalian species. The topo IIα promoter does not contain a consensus TATA motif. The most conserved feature of this promoter is that it contains multiple CCAAT motifs, which are located mostly in inverted orientation. The activity of the promoter is regulated by various external stimuli, such as heat shock, growth arrest, and stages of the cell cycle, and also by the p53 tumor suppressor protein. Studies have shown that the CCAAT motifs play a critical role in the transcription regulation of this promoter by these various agents (3–6).

In a recent study (7), we showed that the mammalian heterotrimeric CCAAT binding factor (CBF) regulates expression of the cellular topo IIα gene as well as transcription activity of the topo IIα promoter. We also showed that inactivation of CBF in mouse fibroblast cells by expression of a dominant-negative CBF subunit results in retardation of cell growth. Analysis of the mRNA profile showed that the inactivation of CBF in fibroblasts decreases the expression of only a small number of cellular genes, including topo IIα and E2F1, which are regulated during cell growth. The results implied that CBF might be primarily involved in transcription of growth-regulated genes in cultured mammalian cells. Because the promoters of various growth-regulated genes, such as topo IIα, cyclin B1, CDC25C, E2F1, and thymidine kinase, are activated at different stages of the cell cycle and contain multiple CBF binding sites (8–12), we hypothesized that these promoters are highly dependent on CBF activity in vitro. One paradox of this hypothesis is that the DNA binding activity of CBF is unchanged during the cell cycle; thus, it remains unclear how constitutively expressed CBF is involved in regulating transcription of various promoters in specific cell-cycle stages.

We recently analyzed the transcription activity of the topo IIα promoter using an in vitro reconstituted nucleosomal as-

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Assembled DNA template (13). The study showed that binding of CBF to the nucleosomal topo IIα promoter disrupts the regular nucleosomal structure over the CBF binding sites as well as over the downstream promoter region containing the transcription start site. Interestingly, binding of CBF to the nucleosomal topo IIα promoter strongly activated transcription. The study also revealed that although the topo IIα promoter contains three major transcription start sites, CBF activates transcription primarily through one of them. The CBF-mediated activation requires activation domains of CBF, which, however, do not play any role in the CBF-mediated nucleosomal disruption. When a mutant nucleosomal promoter containing mutations in all CBF binding sites was used in the in vitro transcription reaction, no transcription from any of the three start sites was observed. This observation suggested that binding of CBF to the topo IIα promoter disrupts the nucleosomal structure, which allows transcription from all three start sites, whereas the activation domains of CBF stimulate transcription through only one of the start sites. Altogether, the results of the study indicate that CBF controls topo IIα promoter activity by two mechanisms, nucleosomal disruption and direct transcription activation. However, the mechanism by which CBF controls activation of the topo IIα promoter during the cell cycle remains to be determined.

In the present study, we show that mutations in all CBF binding sites located in inverted but not in direct orientation that are present in the topo IIα promoter completely abolish transcription activation of the promoter during cell-cycle progression. Analysis of the promoter in an in vitro reconstituted transcription system shows that CBF activates transcription of topo IIα regardless of the orientation of the CBF binding site and controls transcription of only one of the three start sites present in the promoter. Mutations of the transcription start site completely abolish transcription activation by recombinant CBF in vitro but do not affect activation of the promoter during the cell cycle. Two CBF-B mutants, one containing a truncated CBF-B lacking transcription activation domain and the other containing an allele-specific CBF-B mutant, were expressed in mouse fibroblast cells to determine the role of CBF in the cell cycle-dependent transcription activation. This study reveals that the cell cycle-dependent activation of the topo IIα promoter is exclusively dependent on CBF binding but not on CBF-dependent transcription activation mediated by the glutamine-rich domains of CBF.

MATERIALS AND METHODS

Plasmids—To construct the 7CCAAT reporter plasmid, the sequence between −250 and +110 of the mouse topo IIα gene promoter (6) was amplified by PCR and cloned between the SacI and XhoI sites of the pG3-basic vector. The reporter plasmid constructs 4CCAAT, 3CCAAT, and 2CCAAT with deletions at the 5’ end of the topo IIα promoter were generated by PCR using the 7CCAAT template. The constructs W12, W34, W1, W2, W3, W4, and M1–4 harboring point mutations (CCAAT to CCAAA) in the CBF binding sites were obtained by PCR using the W3 template. The constructs −170/+29, −170/+16, −170/+4, and −170/−18 with deletions at the 3’ end of the topo IIα promoter were generated by PCR using the 4CCAAT template. The constructs M−15/−13, M−11/−9, M−8/−6, M−5/−3, M−2/+1, and M+2/+4 carrying nucleotide substitutions in the start site II region were obtained by PCR using −170/+4 as a template. Two plasmid constructs, pTRE-FLAGBmut307 and pTRE-FLAGBmut307, which carry a deleted mutant and an amino acid substituted mutant of the CBF-B subunit, were generated by PCR using the pTRE-FLAGB as template as reported before (7). The reporter construct FC1 containing four CBF binding sites of mouse α2 (1) collagen promoter was described previously (14).

Cell Culture and Transfection—Mouse fibroblast NIH3T3 cells were transfected with plasmids using the Lipectofamine reagent (Invitro-gen), and expression of the luciferase gene was measured as described previously (7). For the experiments illustrated in Figs. 8 and 9, a mouse fibroblast cell line, 3T3TtA, expressing tetracycline-responsive transcription activator (TRES), which was generated in a previous study (7), was used for DNA transfection. To generate a stably integrated CCAAT reporter plasmid into NIH3T3 cells, both 7CCAAT and ptk-Hyg plasmids (9:1 ratio) were first transfected, and then cells were selected in the presence of 200 μg/ml hygromycin. Each of the topo IIα-promoter-luciferase plasmid constructs were analyzed by a minimum of three independent DNA transfection experiments, and an average reporter activity with standard deviations was calculated from results of all the experiments, which are shown in tables, which are described previously (7). To analyze the activity of the reporter constructs during the cell cycle, fibroblast cells were first transfected with plasmids, and then 24 h after transfection, cells were starved with 0.5% serum for 48 h and subsequently activated with 10% serum.

Recombinant CBF Proteins—Full-length and truncated recombinant CBF subunits were generated as fusion proteins with glutathione-S-transferase and purified as described previously (15). The mutant CBF-B subunit (Bmut307) was expressed as a fusion with His tag and was purified as described before (16). For the experiment in Fig. 9C, the wild-type and the mutant CBF-B (But and Bmt307) were expressed in the 3T3TtA fibroblast cell line after transient transfection of either pTRE-FLAG or pTRE-FLAGBmut307 plasmid construct. Each of the expressed CBF-B polypeptides was purified from fibroblast cellular extracts using anti-FLAG-agarose affinity resin (Sigma). Each CBF-B was copurified with the cellular CBF-A and CBF-C subunits present in the fibroblast cells.

In Vitro Transcription—The in vitro transcription reactions were performed using nuclear extracts prepared from HeLa cells. Preparation of nuclear extracts, condition of the in vitro transcription reactions, and subsequent analysis of RNAs using the primer extension method were done as described previously (17).

DNA Binding—The binding of CBF with the topo IIα promoter was analyzed using both electrophoretic mobility shift assay and DNase I footprinting methods as described previously (18). For DNase I footprinting analysis shown in Fig. 3, a topo IIα promoter fragment was isolated from either the 4CCAAT or the M1–4 construct by SacI and XhoI digestion, labeled at the XhoI end using Klenow, and then used in the DNA binding reactions. For the experiment in Fig. 4, 35-30-duplex-stranded oligonucleotides w1, w2, w3, and w4 corresponding to the CCAAT motifs located at −26, −46, −66, and −117 (respectively) in the topo IIα promoter were synthesized, labeled using Klenow, and then used in the DNA binding reactions. In the w1, w2, w3, and w4 oligonucleotides, the CCAAT motif is located in the middle and is flanked by 15-bp topo IIα promoter sequences on both sides. Nuclear extracts used for DNA binding were prepared according to a method described by Schreiber et al. (19).

RESULTS

Topo IIα Promoter but Not Collagen Promoter Containing Multiple CCAAT Motifs Is Activated During the Late S and G2/M Phases of the Cell Cycle—Previously, analysis of the mouse topo IIα promoter showed that it contains seven CCAAT motifs that are binding sites of transcription factor CBF/NF-Y (6, 13). To understand the role of the CCAAT motifs in transcription during the cell cycle, we compared the activity of the topo II promoter with that of a collagen promoter, which contains four CBF binding sites, during cell cycle progression in mouse fibroblasts. In previous studies, we showed that the collagen promoter is strongly activated by CBF in an in vitro reconstituted transcription system (14, 17). The transcription activity using the topo IIα promoters in NIH3T3 cells was determined after either the promoter was stably integrated in chromosome or the promoter construct was transiently transfected in fibroblast cells. The cells were first synchronized in a quiescent state (G0/G1) by serum starvation, and cell-cycle progression was initiated by the addition of serum. The state of synchronization and cell-cycle progression was analyzed by flow
cytometry methods using both propidium iodide and bromodeoxyuridine labeling. The results showed that after serum starvation, about 85% of fibroblast cells enter into G0/G1 phase and that the induction of S phase starts at 12 h and reaches the maximum level at 16 h after serum stimulation, similar to what was seen in our earlier studies (7). The activity of stably integrated topo II\(^{\alpha}\)/H9251 promoter, which is very low in quiescent cells, increased 5-fold at 18 and 24 h after serum addition (Fig. 1A). Similarly, the activity of transiently transfected topo II\(^{\alpha}\) promoter was increased about 6-fold at 18 and 24 h after serum addition. This result shows that the activity of the topo II\(^{\alpha}\)/H9251 promoter is induced at late S phase to G2/M phase. This observation is in good agreement with earlier studies of both human and mouse topo II\(^{\alpha}\) promoters (1, 6). In contrast, the transcription activity of the collagen promoter was not induced after serum stimulation (Fig. 1B). Instead, the collagen promoter activity was lower at late S and G2/M phase compared with the activity of quiescent phase cells before serum stimulation. This indicates that although both the topo II\(^{\alpha}\) and the collagen promoters contain multiple CCAAT motifs, the cell cycle-dependent stimulation of promoter activity is specific to the topo II\(^{\alpha}\) promoter.

**Role of Multiple CCAAT Motifs in Transcription Activation of Topo II\(^{\alpha}\) Promoter during Cell Cycle Progression**—To determine the role of multiple CCAAT motifs in the topo II\(^{\alpha}\) promoter activity during cell-cycle progression, we generated several deletion promoter constructs containing decreasing numbers of CCAAT motifs (Fig. 2A). The activity of the deleted constructs was analyzed during the cell cycle after transient transfection. This shows that the promoter containing four CCAAT motifs (4CCAAT) is activated very similar to the promoter containing seven CCAAT motifs during the cell cycle (Table I). The promoter containing three CCAAT motifs (3CCAAT) is also activated during the cell cycle but to a lesser extent than the promoter containing either four or seven CCAAT motifs. In contrast, the promoter containing two CCAAT motifs (2CCAAT) is activated very little. This shows that the four proximal CCAAT motifs in the topo II\(^{\alpha}\) promoter are required for a high level of promoter activation during the cell cycle.

To determine the role of individual CCAAT motifs in the promoter activity, single nucleotide substitution mutation was
introduced in each of the four CCAAT motifs in the 4CCAAT topo IIα promoter construct (Fig. 2B). The interaction of recombinant CBF with both wild-type 4CCAAT promoter and a mutant M1–4 promoter containing mutations in all four CCAAT motifs was analyzed by the DNase I footprinting method. This shows that CBF interacts with all four CCAAT motif regions in the 4CCAAT promoter (Fig. 3, lanes 1 and 2). In contrast, no interaction of CBF with the M1–4 promoter was observed (lanes 3 and 4), indicating that single nucleotide substitution mutation in each of the four CCAAT motifs results in complete abolition of CBF binding. When a mutant promoter containing a single wild-type CCAAT motif at various locations (W1, W2, W3, or W4) was analyzed, CBF only interacted with the respective wild type but not with the mutant sites (data not shown).

In this regard, it is important to mention that the CBF binding sites in the W1, W3, and W4 promoters are located in inverted orientation as ATTGG, whereas the site in W2 is located in direct orientation.

Analysis of promoter activity for each of the mutants shows that each of the three promoters, W1, W3, and W4, containing a single CBF binding site in the inverted orientation is activated during the cell cycle in a manner very similar to the 4CCAAT promoter (Table I). In contrast, the M1–4 promoter containing mutations in all four CBF sites was not activated during the cell cycle. Interestingly, the W2 promoter containing a single CBF site in the direct orientation was not activated during the cell cycle. This result indicates that at least one CBF binding site in the inverted orientation but not in the direct orientation is required for cell cycle-dependent activation of the topo IIα promoter.

It is possible that binding of CBF to the inverted CCAAT motif may have increased during the cell cycle and that this contributes to the activation of the topo IIα promoter. To test this possibility, we prepared nuclear extracts from fibroblast cells at 0, 12, 18, and 24 h after serum stimulation. Four double-stranded oligonucleotides, w1, w2, w3, and w4, corresponding to each of the CCAAT motifs located at the −26, −46, −66, and −117 positions, respectively, in the topo IIα promoter were made, labeled, and used in the DNA binding assay. This shows that the CBF binding activity in fibroblast nuclear extracts before serum stimulation (0 h) binds to each of the CCAAT motifs with indistinguishable affinity (Fig. 4), indicating that all four CCAAT motifs in the 4CCAAT promoter interact with CBF with similar affinity. Comparison of CBF binding activity at various time points after serum stimulation shows that a smaller increase of CBF binding to the w3 CCAAT motif is observed 18 h after serum stimulation. However, no significant alteration of CBF binding to the w1, w2, or w4 CCAAT motifs was observed at different time points of serum stimulation. This result indicates that activation of the topo IIα promoter during the cell cycle is not caused by an increase of CBF binding to the inverted CCAAT motifs in the promoter.

Analysis of Topo IIα Promoter Constructs in an in Vitro Reconstituted Transcription System—It is possible that CBF-dependent transcription activation of the 4CCAAT promoter may be regulated during the cell cycle. Our previous study showed that although the 4CCAAT promoter contains three

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**TABLE I**

**Activity of a collagen promoter and various deletion and nucleotide substitution mutants of the topo IIα promoter during the cell cycle**

Each of the promoter constructs was analyzed after transient transfection in mouse fibroblast cells followed by starvation with a 0.5% serum concentration and subsequent stimulation with a 10% serum.

| Plasmid Constructs | Relative promoter activity at different time points after serum stimulation | Fold induction |
|--------------------|--------------------------------------------------------------------------|---------------|
|                    | 0 h     | 12 h   | 18 h   | 24 h   |                |
| 7CCAAT             | 12.2 ± 0.6 | 14.4 ± 1.1 | 60.7 ± 4.3 | 92.0 ± 5.7 | 7.4          |
| 4CCAAT             | 13.9 ± 1.0 | 11.0 ± 0.7 | 58.0 ± 4.2 | 100.0 ± 7.2 | 7.2          |
| 3CCAAT             | 5.5 ± 0.3  | 3.7 ± 0.1  | 6.6 ± 0.2  | 20.9 ± 0.9  | 3.8          |
| 2CCAAT             | 3.2 ± 0.1  | 2.7 ± 0.1  | 3.5 ± 0.0  | 5.6 ± 0.3   | 1.7          |
| W12                | 5.4 ± 0.3  | 4.6 ± 0.3  | 21.9 ± 1.8 | 30.7 ± 2.9  | 5.7          |
| W4                 | 16.1 ± 1.1 | 13.3 ± 1.2 | 62.5 ± 3.7 | 99.3 ± 7.8  | 6.2          |
| W1                 | 8.2 ± 0.5  | 7.0 ± 0.4  | 20.2 ± 1.2 | 30.0 ± 2.2  | 3.7          |
| W2                 | 3.1 ± 0.1  | 3.1 ± 0.2  | 2.8 ± 0.1  | 3.4 ± 0.3   | 1.1          |
| W3                 | 9.7 ± 0.4  | 8.0 ± 0.4  | 30.4 ± 1.6 | 46.0 ± 3.0  | 4.7          |
| W4                 | 4.1 ± 0.2  | 2.4 ± 0.0  | 4.7 ± 0.2  | 9.1 ± 0.5   | 2.4          |
| M1–4               | 1.3 ± 0.0  | 1.0 ± 0.1  | 0.7 ± 0.1  | 0.6 ± 0.0   | 0.46         |
| FC1                | 18.3 ± 1.1 | 15.5 ± 0.9 | 11.4 ± 0.5 | 9.4 ± 0.5   | 0.5          |
major start sites, recombinant CBF activates transcription mostly through one of the start sites of the nucleosomal promoter template (13). To understand CBF-dependent topo IIα promoter activity, we analyzed 7CCAAT and 4CCAAT promoters in an in vitro reconstituted transcription system. In a previous study, expression of a mutant CBF-B, Bmut, which inhibits DNA binding of cellular CBF, decreases expression of the topo IIα gene in fibroblasts (7). The recombinant Bmut polypeptide was expressed and purified from bacteria (16). As expected, the addition of recombinant Bmut to HeLa cell nuclear extracts inhibited DNA binding of cellular CBF (Fig. 5A).

Analysis of transcription reactions containing either the 7CCAAT promoter or the 4CCAAT promoter shows that each of the promoters is transcribed from three different start sites, which are designated I, II, and III (Fig. 5B, lanes 1 and 5). Interestingly, the addition of increasing amounts of recombinant Bmut in the transcription reactions results in almost complete inhibition of transcription from start site II but not from start sites I and III of both the promoters (Fig. 5B, lanes 2–4 and 6–8). Consistent with this observation, analysis of transcription from the mutant M1–4 promoter shows that almost no transcription from start site II is observed, whereas start sites I and III are transcribed similar to the wild-type 4CCAAT promoter. Altogether, these results indicate that transcription from start site II of the topo IIα promoter in vitro is dependent on CBF binding to the promoter.

Consistently, the addition of purified recombinant CBF in the in vitro transcription reaction results only in activation of start site II (Fig. 6A, lanes 1 and 2). We have also determined that transcription of the topo IIα promoter occurred in the presence of three truncated forms of CBF; one contains a truncated CBF-B that lacks amino-terminal glutamine-rich domain, the second is composed of truncated CBF-C, which lacks carboxyl-terminal glutamine-rich domains, and the third contains both truncated CBF-B and CBF-C. We previously showed that these three truncated forms of CBF interact with DNA similar to wild-type CBF (17). The addition of the first two forms of truncated CBF containing either truncated CBF-B or truncated CBF-C in the in vitro transcription reactions resulted in activation of transcription from the start site II (lanes 3 and 4). However, the level of transcription activation by these two truncated CBFs was much lower (3- and 2-fold lower, respectively) compared with the transcription activation by the wild-type CBF. In contrast, the addition of the third form of CBF containing both truncated CBF-B and CBF-C resulted in no activation from start site II (lane 5). These results indicate that the two glutamine-rich activation domains of CBF-B and CBF-C subunits mediate transcription activation of the topo IIα promoter through start site II in vitro. The four mutants of the topo IIα promoter, W1, W2, W3, and W4, each of which contains a single CBF binding site, are also analyzed in the in vitro transcription reaction. This shows that the addition of recombinant CBF in the transcription reaction results in activation of each of the promoters specifically from start site II, similar to the wild-type 4CCAAT promoter (Fig. 6B). Altogether, these results indicate that CBF activates the topo IIα promoter in vitro mostly through a specific start site and that
the in vitro CBF-mediated activation is not dependent on orientation or location of the CBF binding site in the topo IIα promoter.

**Mutational Analysis of the Transcription Start Site II Region**—Each of the three start sites of the topo IIα promoter likely contains a basal promoter element. Because none of the start site regions contain a consensus TATA motif, it is most likely that these basal promoter elements contain binding sites for initiator protein. It is not known whether all the start sites of the topo IIα promoter are utilized in the in vivo transcription reaction. Each of the promoter templates is transcribed in vitro in the absence (lanes 1, 3, 5, and 7) and in the presence of full-length CBF subunits (lanes 2, 4, 6, and 8). The recombinant CBF (rCBF) contains 551 ng of full-length CBF-B and 450 ng of full-length CBF-A/CBF-C. The transcription reactions in both A and B are analyzed according to the method described in Fig. 5B.

![Figure 6](image)

**Fig. 6.** A, analysis of the 4CCAAT topo IIα promoter in the in vitro transcription reaction in the presence of full-length and truncated recombinant CBF subunits. The transcription reactions are performed in the presence of 551 ng of the full-length CBF-B subunits (lanes 2 and 4), 342 ng of CBF-Bd lacking amino-terminal glutamine-rich domain of CBF-B (lanes 3 and 5), 450 ng of full-length CBF-A/CBF-C subunits (lanes 2 and 3), and 350 ng of CBF-A/CBF-Cd lacking the carboxy-terminal glutamine-rich domain of CBF-C. B, analysis of the W1, W2, W3, and W4 topo IIα promoter templates in the in vitro transcription reaction. Each of the promoter templates is transcribed in vitro in the absence (lanes 1, 3, 5, and 7) and in the presence of full-length CBF subunits (lanes 2, 4, 6, and 8). The recombinant CBF (rCBF) contains 551 ng of full-length CBF-B and 450 ng of full-length CBF-A/CBF-C. The transcription reactions in both A and B are analyzed according to the method described in Fig. 5B.

**Fig. 7.** The role of the start site II region of the topo IIα promoter in the CBF-dependent transcription activation in vitro. A and B, schematic diagram of the reporter constructs containing deletions in the 3′ end region (A) and nucleotide substitution mutations in the start site II region (B) of the topo IIα promoter. C and D, each of the deletions and the substitution mutants are analyzed in the in vitro transcription reaction with or without the addition of recombinant full-length CBF (rCBF) as described in Fig. 6B. Arrows show the transcripts from each start site for each of the constructs.
Activation of Topo IIα Promoter by CBF during Cell Cycle

Relative promoter activity during the cell cycle for each construct was determined as described in Table I. The promoter activity in the unsynchronized fibroblast cells was determined 48 h after transient transfection. The fold increase of each promoter at 24 h compared with its activity at 0 h after serum stimulation is shown.

+16, and −170/+4, but not −170/−18, are activated during the cell cycle (Table II; data not shown for the −170/+29 and −170/+16 promoters). Altogether, these results indicate that the nucleotide sequences in the start site II region of the topo IIα promoter are essential for both the CBF-dependent transcription activation in vitro and the activation of the promoter during the cell cycle in fibroblast cells.

For further analysis, several mutants containing nucleotide substitutions in the start site II region were generated in the −170/+4 promoter (Fig. 7B). These mutants are also analyzed by the in vitro transcription reaction assay and also during the cell cycle in fibroblast cells. This shows that similarly to the −170/+4 promoter, the mutant promoters m−5/−3, m−11/−9, and m−8/−6, which are transcribed from both start sites I and II, are activated by CBF in vitro (Fig. 7D, lanes 1–8). In contrast, the mutant promoters m−5/−3, m−2/+1, and m+2/+4, which are transcribed only from start site I, are not activated by CBF in vitro (lanes 9–14). Surprisingly, analysis of promoter activity during the cell cycle shows that the mutant promoters m−5/−3, m−2/+1, and m+2/+4, but not m−11/−9 or m−8/−6, are activated during the cell cycle (Table II). These results demonstrate that the nucleotide sequences between −5 to +4, which are important for CBF-mediated transcription activation in vitro, are dispensable for cell cycle-dependent transcription activation in fibroblast cells. Furthermore, this study identifies a promoter element between −11 and −6 that is required for the cell cycle-dependent activation but not for CBF-dependent activation in vitro. It is important to note that although the m−5/−3, m−2/+1, and m+2/+4 promoters are not activated by CBF in vitro, mutations of the CBF binding sites in these promoters abolish the cell cycle-dependent activation in fibroblast cells (data not shown). We interpret the results as indicating that CBF binding, but not conventional CBF transcriptional activation domains, is required for the cell cycle-dependent activation of the topo IIα promoter in fibroblast cells.

Evaluation of CBF Binding and CBF-dependent Transcription Activation in the Topo IIα Promoter Activity in Fibroblast Cells—It is possible that mutations of the CBF binding sites in the topo IIα promoter might inhibit binding of other transcription factors that could result in abrogation of cell cycle-dependent activation in fibroblasts. To determine whether CBF-mediated transcription activation plays a role in the topo IIα promoter activity in fibroblast cells, we have expressed a truncated form of CBF-B lacking a glutamine-rich activation domain under control of a tetracycline-inducible promoter (Fig. 8A). Nuclear extracts prepared from fibroblast cells expressing truncated CBF-B were used in a DNA binding experiment with the labeled W1 oligonucleotide. As a control, nuclear extracts were also prepared from cells transfected with either vector alone or vector expressing the full-length CBF-B subunit. The DNA binding of the control nuclear extracts resulted in generation of a specific CBF-DNA complex (Fig. 8B, first and third lanes). In contrast, DNA binding of nuclear extracts containing the truncated CBF-B forms an additional faster moving complex (second lane). The addition of anti-CBF-B antibodies in the DNA binding reaction containing truncated CBF-B resulted in concomitant inhibition of the faster mobility complex and formation of a supershift complex. We interpret this result as indicating that the faster mobility complex is formed due to the binding of truncated CBF-B together with cellular CBF-A/ CBF-C. When truncated CBF-B is cotransfected with the 4CCAAT topo IIα promoter in fibroblast cells, no change of topo IIα promoter activity was observed (Fig. 8C). In contrast, co-transfection of the truncated CBF-B with the collagen promoter (FC1) resulted in an almost 4-fold inhibition of promoter activity. This result indicates that the transcriptional region of CBF-B is required for activity of the collagen promoter but is dispensable for activity of the topo IIα promoter in fibroblast cells.

To address whether CBF binding to the topo IIα promoter is required for transcription activation during the cell cycle, we have generated an allele-specific mutant of CBF-B, Bmt307 (Fig. 9A). Previous studies of HAP2, the yeast homologue of CBF-B, showed that a mutant HAP2 containing mutation of a lysine to leucine in the DNA binding domain interacts with a high affinity to a mutant CBF site with a CCAAC motif (20). The wild-type CBF interacts very weakly to DNA containing the CCAAC motif. The Bmt307 mutant was generated by a similar mutation from lysine to leucine at the corresponding position in the DNA binding domain of CBF-B. To examine DNA binding activity in vitro, Bmt307 was expressed in bacteria and purified as described earlier for the wild-type CBF polypeptide (17). As expected, purified Bmt307 together with the wild-type CBF-A and CBF-C interacts strongly with DNA containing CCAAC but not DNA containing the CCAAT motif (data not shown).

To test the transcription activity of Bmt307, the mutant was expressed under the control of the tetracycline-inducible promoter in fibroblast cells. We have also generated a mutant W3 promoter (W3mt) containing a single nucleotide substitution mutation of ATTGG (CCAAT) to GTTGG (CCAAC) (Fig. 9B). To examine DNA binding of Bmt307 to the W3mt promoter, we purified the Bmt307 polypeptide that was expressed as a fusion protein with FLAG epitope from fibroblast cell extracts using anti-FLAG immunoprecipitation resin. This purification method allows confirmation of cellular CBF-A and CBF-C together with the Bmt307 polypeptide. The addition of the purified Bmt307 complex in a DNA binding reaction containing the labeled W3mt promoter results in formation of a DNA-protein complex that can be supershifted with anti-CBF-B antibodies (Fig. 9C, lanes 3 and 4). When the DNA binding reaction is performed with a wild-type CBF-B complex that was expressed and purified similar to Bmt307, a DNA-protein complex is formed that...
has similar mobility but much weaker intensity than that with Bmt307 (lane 2). This result indicates that the Bmt307 complex interacts with W3mt with much higher affinity than does the wild-type CBF-B complex. The promoter activity of W3mt is about 3.7-fold lower than that of the W3 promoter in unsynchronized fibroblasts and, unlike the W3 promoter, no increase in promoter activity of W3mt is observed during the cell cycle (Fig. 9D). Interestingly, the expression of Bmt307 results in a 2.7-fold increase in the activity of the W3mt promoter in the unsynchronized fibroblasts and in a 2.3-fold induction of W3mt promoter during the cell cycle. We interpret this result as indicating that the expression of Bmt307 directs formation of a high affinity CBF-DNA complex in the W3mt promoter that results in the induction of W3mt promoter during the cell cycle. Altogether, these results strongly indicate that CBF binding to the topo IIα promoter is essential for activation of the promoter during the cell cycle in fibroblast cells.

FIG. 8. The effect of the expression of a truncated CBF-B lacking an activation domain in fibroblast cells. A, schematic of a truncated CBF-B (Bdbd) containing 240–336 amino acids (aa) of the CBF-B subunit, which was constructed as a fusion with FLAG epitope under control of the tetracycline-inducible promoter (TRE FlagCMV). B, DNA binding of CBF present in nuclear extracts prepared from fibroblast cells transfected with either expression vectors without insert (lane 1) or with Bdbd (lane 2) or with full-length CBF-B (lane 3). The DNA binding was performed with labeled w3 oligonucleotide and then assayed using the electrophoretic mobility shift assay. C, activity of the 4CCAAT topo IIα and the FC1 collagen promoters in fibroblast cells after expression of Bdbd. The promoter activity of each construct was determined after transient transfection in fibroblast cells together with either the expression vector alone (pTRE-FLAG) or with the vector expressing Bdbd polypeptide (pTRE-FlagBdbd). For each construct, the average relative value of three experiments with S.D. is shown.

FIG. 9. The activity of a mutant topo IIα promoter in the presence of an allele-specific CBF-B mutant. A, schematic of a mutant CBF-B, Bmt307, containing single amino acid substitutions from arginine to leucine at position 307, which was constructed as a fusion with the FLAG epitope under control of the tetracycline-inducible promoter. B, schematic diagram of a mutant topo IIα promoter, W3mt, in which the ATTGG (CCAAT) motif at −66 position was mutated to GTTGG (CCAAAC). The CCAAT motifs at −26, −46, and −117 positions were mutated to CCAAA similar to the W3 topo IIα promoter. C, DNA binding of CBF containing Bmt307. The wild-type CBF-B (Bwt) and the mutant Bmt307 were first expressed after transfection in fibroblast cells, and then each of the polypeptides was purified from the fibroblast cell extracts using anti-FLAG antibody affinity resin. Each of the polypeptides was copurified with the cellular CBF-A/CBF-C complex and used in a DNA binding assay with a labeled promoter fragment of W3mt (lanes 2 and 3). The DNA binding reaction in lane 4 contained anti-CBF-B antibody, which was first incubated with the Bmt307 complex and then assayed for DNA binding. D, activity of the W3mt promoter in the presence of Bmt307 in fibroblast cells. The promoter activity was measured with or without expression of the mutant CBF-B, Bmt307, both in the unsynchronized cells and at different time points of serum stimulation of the synchronized cells. Analysis of promoter activity during serum starvation and stimulation was done as described in Table I. The activity of the W3 promoter is shown for comparison. The fold activation of the W3 promoter during the cell cycle is different from that shown in Table I and is possibly due to a different fibroblast cell line.

DISCUSSION

Our results demonstrate that among the multiple CBF binding sites in the topo IIα promoter, a minimum of a single CBF site in the inverted orientation is required for transcription
activation during the cell cycle. The fact that a collagen promoter containing multiple CBF binding sites in the inverted orientation is not activated during the cell cycle is strongly indicative that the role of CBF in transcription activation during the cell cycle is specific to the topo IIα promoter. Analysis of DNA binding shows that CBF interacts very similarly, irrespective of orientation of the CBF binding sites in the topo IIα promoter. We speculate that CBF-dependent transcription activation of the topo IIα promoter is regulated during the cell cycle, and such activation might be specific with respect to the orientation of the CBF binding site. To test this possibility in this study, we have analyzed in more detail the CBF-dependent activation of the topo IIα promoter in vitro. In this regard, our previous study showed that recombinant CBF activated transcription of a nucleosomal topo IIα promoter template in vitro (13). Our present study shows that inhibition of CBF binding to the topo IIα promoter either by mutations in the CBF sites or by a dominant negative CBF-B mutant results in repression of transcription of only one (start site I) of the three start sites present in the topo IIα promoter. This result is consistent with the observation that recombinant CBF activates transcription of the topo IIα promoter in vitro only through start site II but not through the other start sites. These results indicate that CBF-mediated transcription activation only regulates part of the topo IIα promoter activity. This study prompted further analysis of the CBF-dependent start site II. To our surprise, this shows that specific mutations of start site II that completely inhibit CBF-dependent activation in vitro do not affect cell cycle-dependent activation in fibroblast cells. It is important to note that although the mutant promoters with the start site II mutation are not activated by CBF in vitro, the CBF binding sites in these promoters are still required for the cell cycle-dependent activation in fibroblast cells.

To date, the mechanism of CBF-dependent transcription in vivo in a mammalian cell is not clearly understood. Two factors that have restricted our study of CBF in mammalian cells are 1) no mammalian cell line lacking CBF has been isolated, and 2) because of multiple subunits, reconstitution of recombinant CBF in a mammalian cell cannot be done easily. Our earlier studies demonstrated that the association between CBF-B and CBF-ACBF-C heterodimer is reversible. Thus, when a dominant negative CBF-B mutant was expressed in fibroblasts, it formed an inactive complex with the cellular CBF-A/CBF-C by displacing the cellular CBF-B subunit from the CBF protein (7). Consistent with the previous studies, our present results show that a truncated CBF-B lacking a glutamine-rich activation domain but containing a subunit interaction and DNA binding domain also interacts with cellular CBF-A/CBF-C, which binds to the CCAAT motif. This allowed us to perform an assay to determine whether the activation domain of CBF-B mediates the transcription activation of the CBF-dependent promoter in fibroblast cells. Interestingly, this experiment shows that the activation domain of CBF-B does not play any role in transcription activity of the topo IIα promoter but it is required for the transcription activity of the collagen promoter in fibroblast cells. This result is in contrast with the in vitro transcription analysis that showed that the activation domain of CBF-B mediated about 60% of the CBF-dependent transcription activation of the topo IIα promoter. Altogether, these results support a model that the transcription activity of the topo IIα promoter in fibroblasts cells is not dependent on the transcription activation function of CBF that is mediated by the two glutamine-rich domains of CBF subunits.

Expression of an allele-specific CBF-B also results in formation of a complex with cellular CBF-A/CBF-C that binds high affinity to a CCAAC motif DNA. Our results show that mutation of CCAAT to CCAAC in the topo IIα promoter decreases promoter activity and inhibits the cell cycle-dependent activation. Interestingly, expression of the allele-specific CBF-B mutant increases promoter activity of the mutant topo IIα promoter and restores activation of the mutant promoter during the cell cycle in fibroblast cells. Thus, this observation is in good agreement with a conclusion that CBF binding to the topo IIα promoter is essential for the cell cycle-dependent activation of the promoter in fibroblast cells.

It is not clear why CBF binding but not CBF-mediated transcription activation is utilized for the cell cycle-dependent activation of the topo IIα promoter. We speculate that because the activity of CBF is present throughout all stages of the cell cycle, the transcription activation function of CBF should be regulated to avoid constitutive activation of the topo IIα promoter during the cell cycle. Indeed, the data in Table II indicate that the basal promoter element at start site I of the topo IIα promoter that is not responsive to CBF-dependent activation in vitro is utilized in the cell cycle-dependent transcription. This indicates that the activation function of CBF is differentially regulated by the basal promoter elements present in the topo IIα promoter. Because none of the basal promoter elements of the topo IIα promoter contains the TATA motif, it is likely that the differential function of the basal elements is due to binding of different initiator proteins. It is also possible that the specific location and architecture of the basal element in the promoter may play a role in mediating CBF-dependent transcription activation. Recent studies of two adenovirus promoters showed that the basal promoter sequences surrounding the TATA and the initiator elements play an important role in the responsiveness of the promoter toward the upstream activator (21). This suggests that besides an assembly site for the general transcription factor, the basal promoter elements might also contribute a physiologic response that leads to temporal transcription activation. Our study also underscores that the basal promoter structure plays an important role in CBF-dependent transcription activation. The fact that the majority of basal promoter elements of topo IIα are not responsive to direct transcription activation by CBF suggests that this may be a mechanism by which the activity of the topo IIα promoter is dependent only on CBF binding but not on the activation function of CBF. It is not known whether CBF interacted in vivo to the topo IIα promoter during all stages of the cell cycle. However, the in vivo interaction of CBF to the cyclin B1 promoter, which is also activated during the late S and G2/M phases of the cell cycle and whose activity is also dependent on the CBF binding sites in the promoter, was recently studied using the chromatin immunoprecipitation method (12). The result was that CBF interacts with this promoter during all stages of the cell cycle in vivo irrespective of activation of the promoter, implying that the activity of the cyclin B1 promoter during the cell cycle is regulated by the CBF binding but not by the activation function of CBF.

It is possible that binding of CBF to the topo IIα promoter results in formation of an active promoter structure which may allow binding of other transcription factors that leads to activation of the promoter during the cell cycle. Indeed, our recent study showed that binding of CBF to the nucleosomal topo IIα promoter disrupts the regular nucleosomal structure not only in the promoter region containing CBF binding sites but also in a large portion of the downstream promoter region that does not contain the CBF binding site (13). Interestingly, binding of a truncated CBF lacking the two glutamine-rich activation domains also similarly disrupted regular nucleosomal structure in the topo IIα promoter, indicating that the CBF-mediated nucleosomal disruption does not require the activation
domains of CBF subunits. Altogether, the results of our previous studies strongly indicated that only CBF binding, not CBF-mediated transcription activation to the topo IIα promoter, is essential for disruption of the nucleosomal structure in the topo IIα promoter. Although a direct relationship between CBF-mediated nucleosomal disruption and transcription activation of the topo IIα promoter was not established, comparison of the in vitro transcription activity of the nucleosomal 4CCAAT and M1–4 promoters indicated that mutation of the CBF binding sites results in inhibition of transcription through all three start sites in the topo IIα promoter (13). This indicated that in a chromatin promoter template, binding of CBF to the topo IIα promoter is required to maintain transcription from all the start sites in the promoter irrespective of whether the start sites are activated by CBF. This implies that CBF-mediated nucleosomal disruption is a predominant mechanism by which CBF controls transcription of the topo IIα promoter.

Our present study demonstrates that interaction of CBF to the binding site located in inverted but not in direct orientation in the topo IIα promoter leads activation of the promoter during the cell cycle. The orientation-specific CBF function is possibly due to asymmetric CBF binding to the CCAAT motif. Our previous analysis of CBF-DNA interaction using the photocovalently-linked method showed that all the three CBF subunits interact at the 5′ side of the CCAAT motif, whereas only CBF-B and CBF-C, but not CBF-A, interact at the 3′ side (16). This finding indicates that the contact between CBF and the CCAAT motif is asymmetric. In this regard, CBF-mediated transcription activation in vitro, which is mediated by the two glutamine-rich segments of CBF subunits, is not dependent on the orientation of the CBF binding site, suggesting that the orientation-specific CBF function mediates by regions outside the activation domains of CBF subunits, possibly through the conserved segments of the CBF subunits involved to form the CBF-DNA complex. It is also possible that the binding of CBF in the inverted orientation may differentially disrupt the nucleosomal structure in the topo IIα promoter. Indeed, our analysis of CBF-mediated nucleosomal disruption showed that CBF binding results in strong disruption, more in the downstream than in the upstream region of the topo IIα promoter (13). In the present study, mutational analysis in the downstream promoter region showed that a GC box element is required for the cell cycle-dependent activation of the topo IIα promoter. This supports the hypothesis that the binding of CBF in the inverted orientation may allow possible cross-talk between CBF and the GC box binding factor, which may lead activation of the topo IIα promoter during the cell cycle.

In summary, our study revealed that CBF regulates transcription of the topo IIα promoter by two separate mechanisms. The first one requires only CBF binding without action of the CBF activation domains; the second utilizes CBF binding followed by transcription activation mediated by the glutamine-rich domains of CBF subunits. Our study demonstrated the first mechanism by which CBF regulates activation of the topo IIα promoter during the cell cycle and suggests that it is possibly a common mechanism by which CBF regulates transcription of other cell cycle-regulated genes.

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REFERENCES
1. Issacs, R. J., Davies, S. L., Sandri, M. I., Redwood, C., Wells, N. J., and Hickson, I. D. (1998) Biochim. Biophys. Acta. 1400, 121–137
2. Withoff, S., De Jong, S., De Vries, E. G. E., and Mulder, N. H. (1996) Anticancer Res. 16, 1867–1880
3. Furukawa, M., Uchiimi, T., Nomoto, M., Takano, H., Morimoto, R. I., Naito, S., Kowano, M., and Kohono, K. (1996) J. Biol. Chem. 271, 10550–10555
4. Wang, Q., Zambetti, G. P., and Sutcliffe, D. P. (1997) Mol. Cell. Biol. 17, 389–397
5. Falck, J., Jensen, P. B., and Sehested, M. (1999) J. Biol. Chem. 274, 18753–18758
6. Adachi, N., Kobayashi, M., and Kayama, H. (1997) Biochem. Biophys. Res. Commun. 230, 105–109
7. Hu, Q., and Maity, S. N. (2000). J. Biol. Chem. 275, 4435–4444
8. Maity, S. N., and de Crombrugghe, B. (1998) Trends Biochem. Sci. 23, 174–178
9. Hao, K.-M., McMahon, S. L., and Farnham, P. J. (1994) Genes Dev. 8, 1526–1537
10. Zwicker, J., Gross, C., Lucibello, F. C., Truss, M., Ehlert, F., Engeland, K., and Muller, B. (1995) Nucleic Acids Res. 23, 3822–3830
11. Cogswell, J. P., Godlevski, M. M., Bonham, M., Bist, J., and Babiss, L. (1995) Mol. Cell. Biol. 15, 2783–2790
12. Scarlata, S., Gurtner, A., Masi, I., Fontemaggi, G., Der, A., Sacchi, A., Ozato, K., and Piaggio, G. (2001) EMBO Report. 2, 1018–1023
13. Crousy, F., Hu, Q., de Crombrugghe, B., and Maity, S. N. (2001) J. Biol. Chem. 276, 40621–40630
14. Crousy, F., Hu, Q., de Crombrugghe, B., and Maity, S. N. (1995) J. Biol. Chem. 270, 468–475
15. Sinha, S., Kim, J.-S., Sohn, K.-Y., de Crombrugghe, B., and Maity, S. N. (1996) Mol. Cell. Biol. 16, 328–337
16. Liang, S. G., and Maity, S. N. (1998) J. Biol. Chem. 273, 31590–31598
17. Crousy, F., Maity, S. N., Sinha, S., and de Crombrugghe, B. (1996) J. Biol. Chem. 271, 14485–14491
18. Maity, S. N., Golumbek, P. T., Karsenty, G., and de Crombrugghe, B. (1997) Mol. Cell. Biol. 17, 4619–4628
19. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
20. Xing, Y., Fikes, J., and Guarente, L. (1993) EMBO J. 12, 4647–4655
21. Wolner, B. S., and Gralla, J. D. (2000) Mol. Cell. Biol. 20, 3608–3615