Stable Expression of a Dominant Negative Mutant of CCAAT Binding Factor/NF-Y in Mouse Fibroblast Cells Resulting in Retardation of Cell Growth and Inhibition of Transcription of Various Cellular Genes*

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The heterotrimeric CCAAT-binding factor CBF specifically interacts with the CCAAT motif present in the proximal promoters of numerous mammalian genes. To understand the in vitro function of CBF, a dominant negative mutant of CBF-B subunit that inhibits DNA binding of wild type CBF was stably expressed in mouse fibroblast cells under control of tetracycline-responsive promoter. Expression of the mutant CBF-B but not the wild-type CBF-B resulted in retardation of fibroblast cell growth. The analysis of cell growth using bromodeoxyuridine labeling showed that expression of the mutant CBF-B decreased the number of cells entering into S phase, and also delayed induction of S phase in the quiescent cells after serum stimulation, thus indicating that the inhibition of CBF binding prolonged the progression of S phase in fibroblasts. These results provide direct evidence for the first time that CBF is an important regulator of fibroblast growth. The inhibition of CBF binding reduced expression of various cellular genes including the α2(1) collagen, E2F1, and topoisomerase IIα genes which promoters contain the CBF-binding site. This result implied that expression of many other genes which promoters contain CBF-binding site was also decreased by the inhibition of CBF binding, and that the decreased expression of multiple cellular genes possibly caused the retardation of fibroblast cell growth.

Eukaryotic RNA polymerase II promoters consist of multiple DNA elements that together control the transcriptional activity of the promoters. Among these various elements, several are present in many different polymerase II promoters. In higher eukaryotic promoters, the CCAAT box is most often found between 80 and 100 base pairs upstream of the transcription start site, and the sequences around the CCAAT are required for high-affinity CBF binding and that the CCAAT motifs of various mammalian promoters contain the CBF-binding site. This result implied that expression of many other genes which promoters contain CBF-binding site was also decreased by the inhibition of CBF binding, and that the decreased expression of multiple cellular genes possibly caused the retardation of fibroblast cell growth.

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1 The abbreviations used are: CBF, CCAAT binding factor; PCR, polymerase chain reaction; tTAT, tetracycline-responsive transcription activator; CMV, cytomegalovirus; TRE, tetracycline responsive; BrdUrd, 5′-bromo-2′-deoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; DDRT, differential display reverse transcription.

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have mostly been studied by in vitro transcription assay or by DNA transfection experiments in different cell lines. Generally, mutation in the CBF-binding site results in decreased basal promoter activity. Previous studies of CBF binding in the promoters of serum albumin and major histocompatibility complex class II genes showed that CBF facilitates formation of stable transcription complexes together with tissue-specific transcription factors. This indicated that CBF might regulate the transcription of various promoters by cooperative interaction with promoter-specific transcription factors (18, 19). More recently, studies of the Xenopus hsp70 promoter showed that CBF binding to this promoter disrupts local chromatin structures and facilitates transcription within the chromatin (20). This demonstrated that the Xenopus CBF interacts with p300 acetyltransferase, which modifies chromatin by acetylation, thus suggesting that CBF could regulate the in vivo transcription of various promoters by chromatin modification.

To understand in vivo function of CBF in mammalian cells, in this study a dominant negative CBF-B mutant was expressed stably in mouse fibroblasts under the control of a tetracycline-responsive promoter. The consequent expression of the mutant CBF-B inhibited the DNA binding of cellular CBF and interestingly resulted in the retardation of fibroblast cell growth. Our results also showed that the expression of the mutant CBF-B delayed progression of S phase and reduced the expression of several genes which promoters contain CBF-binding site. This result implied that the retardation of cell growth is possibly caused by decreased expression of various cellular genes.

**Materials and Methods**

**Plasmids**—The pTet-Off vector (CLONTECH) which was originally developed by Gossen and Bujard (21), expresses a tetracycline-responsive promoter consisting of seven copies of a tetracycline operator sequence (TRE) promoter that encodes a tetracycline-responsiveness regulator (TetR) which is expressed at low levels in the absence of tetracycline. In the presence of tetracycline, the TetR protein binds to its operator sites to reduce transcription of downstream genes. To achieve this, a modified multiple cloning site of the pTRE vector was used to clone genes downstream of the TRE promoter, a double-stranded oligonucleotide (upstream of the TRE promoter) that encodes a FLAG peptide epitope (Met-Asp-Tyr-Lys-Asp-Asp-Asp-Lys) was cloned between the NcoI and NdeI sites of the multiple cloning site of the pTRE vector. This modified multiple cloning site contained unique SacII, EcoRV, BglII, NcoI, NdeI, EcoRI, XbaI, and BamHI restriction enzyme sites. A double-stranded oligonucleotide (upper strand: 5’-CATGGACTACAAAGGAGGATGGATATTTCCGAGTAGAAACA-3’; lower strand: 5’-CTAACTTCCGCTCCAGTTGTCCTCCTGGCT-3’) that encodes a FLAG peptide epitope (Met-Asp-Tyr-Lys-Asp-Asp-Asp-Lys) was cloned between the NcoI and NdeI sites of the multiple cloning site. This constructed plasmid was designated pTRE-FLAG.

The DNA probe of mouse topoisomerase II and E2F1 genes for Northern blot analysis were generated by PCR using double-stranded cDNA of mouse embryo fibroblasts as a template. The PCR primers, mtopo 4273 (5’-GAGCTGGTGACATGGAATGGCA-3’) and mtopo 4594 (5’-AATTGCGCTCCTCCTCCGCTCAGATTGTCCTG-3’) were based on the sequence of mouse topoisomerase IIα cDNA (22) and were used to amplify nucleotides 4273–4594 of the topoisomerase IIα cDNA. The PCR primers mE2F 877 (5’-CCCTCTGAGCCCACTACTAAGCTTGTA-GA-3’) and mE2F 1320 (5’-CCCTCTGAGCCCAATGGTGA-TGATGCAAG-3’) were based on the mouse E2F1 sequence (23) and were used to amplify nucleotides 877–1320 of the E2F1 cDNA. The amplified DNA fragments were cloned into the TA cloning vector pCR2.1-TOPO (Invitrogen) and checked by DNA sequencing. The DNA probe of mouse α2(I) collagen gene used in the Northern blot hybridization was described previously (24).

**Cell Culture, Transfection, and Isolation of Stable Fibroblast Cell Lines**—Mouse fibroblasts NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. To select stable cell clones expressing TetR, the NIH3T3 cells were transfected with the pTet-Off plasmid using the calcium phosphate precipitation method (25). Several days after transfection, colonies of the presence of 800 μg/ml G418 were isolated. To examine the expression of TetR in the selected clones, each clone was transfected with the pTRE-Luc plasmid using LipofectAMINE reagent (Life Technologies, Inc.), and expression of the luciferase gene was measured using luciferin as substrate with a luminometer (26). In one cell clone luciferase expression was high when the clone was cultured in the absence of tetracycline but reduced 100-fold when cultured in the presence of 2 μg/ml tetracycline. This clone cell was then subsequently used to establish stable cell clones expressing either the wild-type or the mutant CBF-B protein. In brief, this cell clone was co-transfected with either the pTRE-flagB or the pTRE-flagBmut plasmid together with the pTK-Hyg plasmid in a 9:1 ratio. After transfection, individual cell clones were selected and isolated in the presence of 400 μg/ml G418, 200 μg/ml hygromycin, and 2 μg/ml tetracycline in the culture medium. Each isolated clone was subsequently maintained in culture medium containing 400 μg/ml G418, 100 μg/ml hygromycin, and 2 μg/ml tetracycline. To examine the expression of CBF-B, each cell clone was cultured at high cell density in the presence and absence of tetracycline for 3 days and then subjected to Western blot analysis.

**Western Blot Analysis**—For initial screening to identify cell clones expressing CBF-B, the individual cell clones were grown in 6-well plates in the presence and absence of tetracycline. The cells in each well were washed twice with phosphate-buffered saline; resuspended in 20 ml of buffer containing 50 μg/ml Tris, pH 6.8, 2% SDS, and 100 mM dithiothreitol; and then extracted by incubating in a boiling water bath for 5 min. The extracts were then centrifuged for 5 min at high speed in a microcentrifuge. Next, 10 μl of the supernatant of each cell extract was fractionated on a 12% SDS-polyacrylamide gel and then electroblotted onto a nitrocellulose membrane (Schleicher & Schuell). Both wild-type and mutant CBF-B protein in the blot was detected by the ECL Western blotting detection system (Amersham Pharmacia BioTech) using anti-FLAG M2 monoclonal antibody (Eastman Kodak) that detects subsequent epitopeIconModule.

For quantitative Western blot analysis which is presented in Fig. 2, monolayer fibroblast cultures grown in 100-mm dishes were harvested by trypsin treatment and collected by centrifugation. The cells were then washed twice with phosphate-buffered saline, resuspended in a buffer (40 μl of buffer per 1 × 106 cells) containing 1% sodium deoxycholate, 1% Triton X-100, 50 μg/ml Tris, pH 7.5, 150 mM sodium chloride, 0.1% sodium deoxycholate, 20 μg/ml sodium pyrophosphate, 50 μM sodium fluoride, and 0.5 mM sodium orthovanadate, and lysed by sonication. The protein in the cell extracts was quantified using a DC protein assay kit (Bio-Rad). Thirty micrograms of each cell extract was fractionated on a 12% SDS-polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. The CBF-B protein in each blot was detected by the ECL Western blot method as described earlier.

**Growth Rate Determination, Cell Cycle Analysis, and Serum Stimulation**—To determine cell growth, 2 × 105 cells of each stable clone were seeded onto a 100-mm dish in the absence or presence of 2 μg/ml tetracycline. Fresh culture medium was added to each dish at intervals of 2 days. Cells were collected by trypsinization at different time intervals, stained with trypan blue, and counted with a hemacytometer. This experiment was performed independently three times.

For cell cycle analysis, cells were plated and maintained in the same way as for the growth rate experiment and then analyzed by flow cytometry using propidium iodide staining as described by Tate et al. (27), or analyzed by 5-bromo-2’-deoxyuridine (BrdUrd) labeling to determine the number of proliferating cells (or the cells going through the S phase) in the cell cycle. For flow cytometry analysis, cells were harvested after 72 h in culture by trypsinization, resuspended in 10 ml of Dulbecco’s modified Eagle’s medium containing 20% calf serum, and collected by centrifugation. The cell pellet was then resuspended gently in a solution containing 50 mg/ml propidium iodide, 4 μg/ml sodium citrate, pH 7.5, 30 units/ml boiled RNase, and 0.1% Triton X-100 and incubated at 37 °C for 10 min. After incubation, NucCl was added to the
cell suspension to a final concentration of 0.25 m. Finally, cells were analyzed in a fluorescence-activated cell sorter (FACScan, Becton Dickinson) and the percentages of cells in different phases of the cell cycle were determined by using the Lysys II version 1.2 program. To determine the number of proliferating cells, the cells were labeled with 10 μM BrdUrd for 10 min in culture and the BrdUrd-labeled cells were detected by the immunocytochemical method using the in situ cell proliferation kit, FLUOS (Roche Molecular Biochemicals) and followed by flow cytometric analysis. The details of the incubation procedure are described in the instruction manual. Briefly, after BrdUrd labeling the cells were harvested by trypsinization, resuspended in phosphate-buffered saline, and then fixed with 70% ethanol. The fixed cells were incubated with 4N HCl to denature cellular DNA, and then mixed with 5 volumes of phosphate-buffered saline containing 0.5% Triton X-100 (PBT). The cells were collected by centrifugation, incubated in a solution containing fluorescein-conjugated anti-BrdUrd monoclonal antibody, and then analyzed in a fluorescence-activated cell sorter as described earlier. The percentage of BrdUrd-labeled cells were determined using the Lysys II program.

For serum stimulation experiments, 10^6 cells of each cell clone were seeded in 150-mm dish and grown in culture media with regular serum (10% fetal calf serum) for 48 h and then stimulated for growth in a media containing 10% fetal calf serum after serum stimulation and then analyzed by flow cytometric analysis. The details of the incubation procedure are described in the instruction manual. Briefly, after BrdUrd labeling the cells were harvested by trypsinization, resuspended in phosphate-buffered saline, and then fixed with 70% ethanol. The fixed cells were incubated with 4N HCl to denature cellular DNA, and then mixed with 5 volumes of phosphate-buffered saline containing 0.5% Triton X-100 (PBT). The cells were collected by centrifugation, incubated in a solution containing fluorescein-conjugated anti-BrdUrd monoclonal antibody, and then analyzed in a fluorescence-activated cell sorter as described earlier. The percentage of BrdUrd-labeled cells were determined using the Lysys II program.

For cell death analysis, 2 × 10^5 cells were seeded onto a slide chamber in the absence or presence of tetracycline and grown for 72 h. The apoptotic cell death in the culture chamber was determined by TUNEL reaction (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) using in situ cell death detection kit (Roche Molecular Biochemicals). The details of the procedure are described in the instruction manual. Briefly, after 72 h in culture the cells were fixed with 4% paraformaldehyde, incubated in a permeabilization solution containing 0.1% Triton X-100, and then labeled by TUNEL reaction with a fluorescein-dUTP. The labeled cells were detected using the fluorescein isothiocyanate followed by centrifugation of the cell extracts in a cesium chloride cushion (34). The resulting RNA pellet was resuspended in 0.3 M sodium acetate, pH 6, and then precipitated by ethanol. Ten micrograms of RNA from each cell clone was then separated on a 1% agarose-formaldehyde gel for Northern blot analysis using standard procedures. Briefly, RNAs were transferred to a ZetaProbe blotting membrane (Bio-Rad) and fixed by UV cross-linking. Membranes were hybridized 12–16 h at 65 °C with 1–2 ng of radiolabeled probe/ml in 0.3 M sodium phosphate buffer, pH 7.2, and 7% SDS. The radiolabeled DNA probes were prepared by random priming. After hybridization, the membranes were washed at 65 °C once in 2 × SSC (1 × SSC contained 150 m NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% SDS for 30 min and twice in 0.2 × SSC and 0.1% SDS for 15 min. The radioactive bands in the membranes were detected by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics).

RESULTS

Stable Expression of Wild-type and Mutant CBF-B in Mouse Fibroblast Cells—Previous studies had shown that a DNA-binding domain mutant of CBF-B which interacted with CBF-A/CFB-C but did not bind DNA, inhibited DNA binding of wild-type CBF subunits in vitro. Those studies also indicated that the mutant CBF-B acted as a competitive inhibitor of interaction between wild-type CBF-B and CBF-A/CFB-C and formed an inactive complex with wild-type CBF-A/CFB-C (35, 36). Thus, to inhibit the DNA binding of cellular CBF in vivo, the mutant CBF-B and, as a control, the wild-type CBF-B were expressed stably under the control of a tetracycline-responsive promoter in NIH3T3 cells. Each of the wild-type and mutant CBF-B cDNA was inserted into a pTRE vector as in-frame fusion with DNA encoding theFLAG epitope (Fig. 1). We then used a two-step selection procedure to isolate the stable fibroblast cell clones. In the first step, cell clones that stably expressed the tetracycline-regulated transactivator ITA protein were isolated by transfecting NIH3T3 cells with the pITe-OFF vector. To monitor expression of the ITe protein, the cell clones were transfected with the pITe-Luc vector, which contains a luciferase reporter gene under the control of the tetracycline-responsive promoter. In one cell clone luciferase expression was repressed in the presence of tetracycline but induced 100-fold in the absence of tetracycline (data not shown). In the second step, this cell line was transfected with the CBF-B cDNA constructs. Expression of CBF-B protein in the cell clones was then detected by Western blot analysis using anti-FLAG antibodies. Three cell clones expressing wild-type CBF-B (B-1, B-2, and B-3), and three cell clones expressing mutant CBF-B (Bmut-a, Bmut-b, and Bmut-c) were isolated (Fig. 2). Each of these cell clones expressed CBF-B protein in the absence of tetracycline (lanes 2, 4, 6, 8, 10, and 12), but not in the presence of tetracycline (lanes 1, 3, 5, 7, 9, and 11), indicating that expression of CBF-B in these cell clones is tightly regulated by tetracycline. Nuclear extracts were prepared from each of the cell clones and were examined for CBF binding activity by electrophoretic mobility shift assay (Fig. 3A). As a control, DNA binding activ-
A Dominant Negative Mutant CBF Subunit Inhibits Cell Growth

Inhibition of DNA binding of cellular CBF by expression of mutant CBF-B in Bmut cell clones. Nuclear extracts were prepared from each cell clone which was grown in the presence (+) or absence (−) of tetracycline (Tc) for 60–72 h. Three micrograms of nuclear extract from each clone was added in a DNA binding reaction along with either a radiolabeled oligonucleotide containing the CBF-binding site of the mouse α(2) collagen promoter (A) or a radiolabeled oligonucleotide containing the consensus binding site for the Oct1 transcription factor (B).

Expression of Mutant but Not Wild-type CBF-B Results in Cell Growth Arrest—Each stable cell clone was analyzed for its growth in culture with or without tetracycline. The B-1, B-2, and B-3 cells grew at almost equal rates in the presence and absence of tetracycline, indicating that the expression of excess wild-type CBF-B had no effect on the cell growth rate (Fig. 4A). In contrast, almost no growth of the Bmut-a and Bmut-b cells and little growth of the Bmut-c cells was observed in the absence of tetracycline, while normal growth was seen in the presence of tetracycline (Fig. 4, A and B). This result indicated that the expression of mutant CBF-B, which inhibited the DNA binding of cellular CBF, resulted in inhibition of cell growth. The cell growth of the Bmut-c cells was less inhibited than that of the Bmut-a and Bmut-b cells, which is consistent with the observation that less of the mutant CBF-B protein was expressed in the Bmut-c cells than in the Bmut-a and Bmut-b cells.

To determine the cell cycle distribution of the growth-inhibited cells, we used flow cytometry to analyze the Bmut-b cells grown in the presence and absence of tetracycline. This analysis showed that the cell cycle distributions of Bmut-b growing cells in the presence of tetracycline and of Bmut-b growth-arrested cells in the absence of tetracycline were very similar (data not shown), thus suggesting that the expression of mutant CBF-B in the Bmut cells did not result in growth arrest in a specific cell cycle stage, but likely resulted in growth retardation in all stages of the cell cycle. To detect specifically the number of cycling cells or the S phase cells, we first labeled the Bmut-b cells at different times in culture by short incubation with BrdUrd and then the percentage of BrdUrd-labeled cells was determined using the immunohemical method followed by flow cytometry. The BrdUrd which is a thymidine analogue, incorporated into the genomic DNA of the DNA synthesizing cells (= the S phase cells) but not the resting cells. The percentage of BrdUrd-labeled Bmut-b cells in the absence of tetracycline is significantly lower than that of the BrdUrd-labeled Bmut-b cells in the presence of tetracycline (Fig. 4C), thus indicating that the expression of mutant CBF-B reduced the number of cells entering into S phase.

It is possible that the growth retardation of the Bmut cells in the absence of tetracycline may be caused partly by cell death or apoptosis. To test this possibility we analyzed the Bmut-b cells by in situ cell death detection method using TUNEL reaction (TdT-mediated dUTP nicked labeling) which determines genomic DNA cleavage that generally occurs during apoptosis. In this experiment very few apoptotic cells were observed in the culture chamber containing the Bmut-b cells grown in the presence of tetracycline, and no increase in the...
apoptotic cell numbers were observed in the Bmut-b cells grown in the absence of tetracycline (data not shown). Thus this result indicates that the growth retardation of the Bmut-b cells in the absence of tetracycline is not caused by apoptosis.

To determine cell cycle progression, the B-2 and the Bmut-b cells were first synchronized by serum starvation and then stimulated to grow by adding serum. Analysis of DNA content of the serum-starved cells by flow cytometry demonstrated that about 80% of the cells entered into quiescence state (G0/G1 phase) after the starvation period (data not shown). Nuclear extracts were prepared from cells at different time points after serum addition and assayed for CBF binding activity using electrophoretic mobility shift assay. No change in CBF binding activity was observed in the nuclear extracts of B-2 cells at different time points after serum addition, and as expected, the CBF binding activity was similar in the nuclear extracts of the cells grown in the presence and absence of tetracycline (data not shown). The CBF binding activity in the nuclear extracts of Bmut-b cells in the presence of tetracycline was very similar at different time points after serum addition (Fig. 5, lanes 1, 3, 5, 7, and 9). However, the CBF binding activity in the nuclear extracts of the cells in the absence of tetracycline was strongly inhibited before serum addition and also at each different time point after serum addition (lanes 2, 4, 6, 8, and 10). As a control, the DNA binding activity of CTF/NF-1 which is known in the literature as another CCAAT-binding transcription factor, was measured and found to be unchanged in the nuclear extracts of Bmut-b cells in the presence and absence of tetracycline (lanes 11–20). These results indicate that the mutant CBF-B was expressed in the serum-starved Bmut-b cells and specifically inhibited CBF binding activity before and at different time points after serum addition. The cell numbers were counted at different time points during serum stimulation. This showed that the doubling time of the B-2 clone was very similar (about 28 h) in the presence and absence of tetracycline (data not shown). In contrast, the doubling time of the Bmut-b clone was 26 and 51 h in the presence and absence of tetracycline, respectively (Fig. 6A). To determine S phase of the Bmut-b cells during serum stimulation, we used the BrdUrd labeling method as described earlier. This showed that the
percentage of BrdUrd-labeled Bmut-b cells in the presence of tetracycline started to increase to a high level at 12 h and reached a maximum level at 16 h after serum stimulation (Fig. 6B). In contrast, the percentage of BrdUrd-labeled Bmut-b cells in the absence of tetracycline increased very little at 12 h and reached a maximum level at 20 h after serum stimulation. Furthermore, the maximum level of the BrdUrd-labeled cells in the absence of tetracycline is significantly lower than that of the BrdUrd-labeled cells in the presence of tetracycline. These results indicate that expression of the mutant CBF-B in the Bmut-b cells delayed entry into S phase from G1 phase, and also reduced the percentage of cells entering into S phase.

Identification of Genes whose Expression Is Altered as a Consequence of Inhibition of CBF Binding—Previously using an in vitro reconstituted transcription system we demonstrated that recombinant CBF activates transcription of the \( \alpha_2(1) \) collagen promoter which contained a CBF-binding site, and that mutation in the CBF-binding site of the promoter abolished the CBF-dependent transcription activation (16), indicating that the CBF binding to the promoter is required for the \textit{in vitro} transcription activation of this promoter. The \( \alpha_2(1) \) collagen which is a subunit of the type I collagen, is expressed at high level in fibroblast cells. To analyze expression of this gene in the B and Bmut clones, total RNAs were isolated from these cell clones in the presence and the absence of tetracycline and then used in the Northern blot analysis. As a control expression of the ribosomal protein S6-encoding gene (S6) was measured. The level of expression of the collagen gene is very similar in B-2 or B-3 cells in the presence and the absence of tetracycline (Fig. 7A, lanes 1 and 2; the data for B-2 cells are not shown). In contrast, the collagen gene was expressed about 6-fold lower in the Bmut-b or Bmut-a cells in the absence of tetracycline than in the presence of tetracycline (Fig. 7A, lanes 3 and 4; data for Bmut-b cells are not shown), indicating that the inhibition of CBF binding in the Bmut cells resulted in reduction of expression of the \( \alpha_2(1) \) collagen gene. Thus as consistent with our previous \textit{in vitro} studies, the present analysis indicated that the CBF binding is required for the \textit{in vivo} transcription of the \( \alpha_2(1) \) collagen gene.

**Fig. 6.** Analysis of serum stimulated growth of quiescent Bmut-b cells using the BrdUrd labeling method. The Bmut-b cells were first synchronized in the presence (+) and absence (−) of tetracycline (Tc), and then stimulated to grow by serum addition as described in Fig. 5. A, the cells were harvested at 0, 12, 24, 36, and 48 h after serum stimulation and then counted as described in the legend to Fig. 4A. B, at different time points after serum stimulation the cells were labeled with BrdUrd for 30 min, and the percentage of the BrdUrd-labeled cells was measured using the method described in the legend to Fig. 4C. The solid black bar and empty bar indicate the percentage of BrdUrd-labeled Bmut-b cells in the presence and the absence of tetracycline, respectively.

**Fig. 7.** Inhibition of CBF binding reduced expression of \( E2F1 \), topoisomerase IIa, and \( \alpha_2(1) \) collagen genes. A, total RNAs were isolated from equal numbers of cells of B-3 and Bmut-a clones grown 72 h in the presence and absence of tetracycline. Ten micrograms of each RNA sample was separated on a 1% agarose-formaldehyde gel and analyzed for expression of different genes by the Northern blot method. B and C, B-2 and Bmut-b cells were first synchronized and then stimulated to grow as described in the legend to Fig. 5. The cells were harvested at different time intervals and were used to isolate total RNAs. Ten micrograms of each RNA sample was used in the Northern blot analysis. The total RNAs isolated after 12 and 15 h of serum stimulation were used to detect expression of the \( E2F1 \) gene (B), and the total RNAs isolated after 18 and 21 h of serum stimulation were used to detect expression of the topoisomerase IIa and \( \alpha_2(1) \) collagen genes (C). In each experiment, expression of the S6 gene, which encodes ribosomal S6 protein, was measured as a control. The 18 S and 28 S RNA bands of each loaded RNA sample are shown at the bottom of the figures. The radiolabeled bands were quantitated with a PhosphorImager and adjustments were made for the amount of total RNA loaded in each lane, which was measured in relation to the amount of S6 band and the intensity of 28 S and 18 S RNAs.
Recent studies have indicated that promoters of several genes including E2F-1, topoisomerase IIα (topo IIα), thymidine kinase, and protein phosphatase cdc25C whose transcriptions are regulated during the cell cycle contain multiple CBF-binding sites, and that expression of many of these genes are crucial for cell cycle progression (4, 37–40). For example, the mRNA level of the E2F1 gene, a member of the E2F of family transcription factors, is increased severalfold at the G1/S phase of cell cycle, and the expression of E2F1 in quiescent cells can activate transcription of several G1/S-phase-regulated promoters (38, 41), suggesting that E2F1 plays a critical role in cell cycle progression through G1 to S phase. In the case of topo IIα which is one of the two isoforms of the DNA topoisomerase II enzyme that plays an essential role in several cellular processes such as replication, recombination, and chromosome condensation and segregation (39), the level of its mRNA varies through the cell cycle: low in G1 and increasing through S phase to maximal in G2/M phase. If CBF acts as an upstream positive regulator of transcription of both the E2F1 and topo IIα genes, then inhibition of CBF binding in vivo should result in decreased expression of these critical genes and lead to cell growth retardation. To test this possibility, we analyzed the expression of the E2F1 and topo IIα genes in B-2 and Bmut-b cells. Total RNAs were isolated at different time points after serum addition in the synchronized B-2 and Bmut-b cells grown with and without tetracycline, and then subjected to Northern blot analyses to measure expression of the E2F1 and topo IIα genes. As expected from previous studies, in these cell clones E2F1 and topo IIα were expressed at maximal level at 15 and 21 h after serum addition, respectively (Fig. 7, B and C).

The level of expression of these genes were very similar in the B-2 cells in the presence and absence of tetracycline. In contrast, both the E2F1 and the topo IIα genes were expressed about 2.5-fold lower in the Bmut-b cells in the absence of tetracycline than in the presence of tetracycline. These results indicated that inhibition of CBF binding in the Bmut-b cells resulted in reduction of expression of both E2F1 and topo IIα genes that occurred during cell cycle progression. In this experiment, the expression of the α2(1) collagen gene which transcription is not regulated during cell growth was reduced 6-fold by the inhibition of CBF binding (Fig. 7C), similar to the earlier experiment in the unsynchronized cells in Fig. 7A. Altogether these results showed that the inhibition of CBF binding decreased expression of the E2F1, topo IIα, and α2(1) collagen genes which promoters contained the CBF-binding site, thus implicating that CBF directly regulates expression of these genes in vivo in mouse fibroblasts.

Since the CBF-binding site is present in the promoters of many mammalian class II genes, the inhibition of CBF binding may have altered the transcription of many other cellular genes. To test this possibility, we monitored the mRNA profiles of B-2 and Bmut-b cells using the DDRT-PCR method (42). The DDRT-PCR method is widely used to identify differential gene expression. In brief, total RNAs were isolated from each of the B-2 and Bmut-b cells in the presence and absence of tetracycline, and then used in DDRT-PCRs as described under “Materials and Methods.” The amplified cDNAs generated from the RNAs of the B-2 and Bmut-b cells were separated on a denaturing polyacrylamide gel. The patterns of cDNA bands were then compared to identify differentially expressed genes in the Bmut-b cells grown with and without tetracycline were very similar in intensity (Fig. 8, A–C, lanes 3 and 4). Moreover, these cDNA bands were also comparable in intensity to the cDNA bands of B-2 cells grown with and without tetracycline (Fig. 8, A–C, lanes 1 and 2). These results suggested that the levels of many different mRNAs were not changed significantly by the expression of mutant CBF-B in the growth-inhibited Bmut-b cells. Nonetheless, the DDRT-PCR analysis identified two cDNA bands whose levels were specifically decreased in the Bmut-b cells but not in the B-2 cells grown in the absence of tetracycline (indicated by arrows in Figs. 8, A and B). These cDNA bands were isolated from the gel, reamplified by PCR, subcloned, and then identified by DNA sequencing. The DNA sequences of the two clones thus isolated from the cDNA bands of Figs. 8, A and B, matched completely with the sequences of the Egr1 gene and Mts1 gene, respectively. Subsequent Northern blot analysis showed that the levels of both the Egr1 and Mts1 mRNAs were decreased by about 30 and 65%, respectively, in the Bmut-b cells but not in the B-2 cells in absence versus in the presence of tetracycline (Fig. 8D). This indicated that expression of the Egr1 and Mts1 genes was decreased as a result of the inhibition of CBF binding in the Bmut-b cells. The Egr1 gene (also known as Krox-24) encodes a zinc finger-containing DNA-binding protein and is one of the early growth response genes (32). The Mts1 gene, which encodes a calcium-binding protein, was initially isolated from metastatic tumors in which it was expressed at much higher level than in normal tissue. The Mts1 gene is also expressed in various established cell lines and appears to be regulated during cell growth (31). Altogether our results showed that the inhibition of CBF binding decreased the expression of multiple genes including several apparently cell cycle regulated genes.

**DISCUSSION**

Our results indicated that stable expression of mutant CBF-B inhibits the DNA binding of cellular CBF and thus acts as a dominant negative mutant in vivo. Interestingly, expression of the mutant but not the wild-type CBF-B resulted in arrest of fibroblast cell growth, which in turn correlated well with the inhibition of CBF binding, thus indicating that the DNA binding activity of CBF is required for normal fibroblast cell growth. The nature of the cell growth arrest was revealed by our flow cytometry, BrdUrd labeling, and TUNEL assay analyses. The flow cytometry and TUNEL assay indicated that the growth arrest is not due to either the growth arrest in specific cell cycle stage or the apoptotic cell death. In contrast, the BrdUrd labeling analysis which measured specifically the DNA synthesizing or the S phase cells, showed that expression of the mutated CBF-B resulted in reduction of BrdUrd-labeled cells indicating that the inhibition of CBF binding decreased the number of cells going through the S phase of cell cycle. In the case of serum starvation/stimulation experiment, the mutant CBF-B was expressed in the Bmut-b cells in the absence of tetracycline during the starvation period and inhibited almost 90% of the cellular CBF binding activity in the quiescent cells. The inhibition of CBF binding in the quiescent Bmut cells did not completely inhibit the serum-stimulated cell growth. However, the doubling time of growth of the Bmut cells expressing mutant CBF-B is two times longer than that of the Bmut cells not expressing mutant CBF-B, thus indicating that the inhibition of CBF binding reduced significantly the rate of progression of cell growth. The BrdUrd labeling analysis of the quiescent cells at different times after serum stimulation clearly showed that the expression of the mutant CBF-B delayed the progression of S phase and also reduced the percentage of cells progressing through the S phase. Thus the inhibition of CBF binding results in inhibition of both the rate of progression of S phase and the amount of cells entering into the S phase which is most likely cause of the retardation of cell growth.

The inhibition of CBF binding decreased significantly ex-
Fig. 8. Differential display of mRNAs of B-2 and Bmut-b cells in presence (+) and absence (−) of tetracycline (Tc). Total RNAs were isolated from equal numbers of cells of B-2 and Bmut-b clones grown 72 h in the presence and absence of tetracycline. The mRNA profiles of the total RNAs were monitored by the DDRT-PCR method using an RNAimage kit. First-strand cDNA for each RNA preparation was synthesized by reverse transcription reaction using each of three 1-base anchored oligo(dT) primers (H-T11G, H-T11A, and H-T11C). Each cDNA was then used as a template in a PCR reaction containing the specific oligo(dT) primer which was used to synthesize the cDNA, in combination with each of eight different arbitrarily designed 13-mer oligonucleotides (H-AP1, H-AP2, H-AP3, H-AP4, H-AP5, H-AP6, H-AP7, and H-AP8). The PCR reactions were labeled with [35S]dATP during amplification and then analyzed in a sequencing gel. Each panel of this figure shows a PCR reaction that was generated by the primer combination shown at the bottom of the panel. The arrows in panels A and B indicate the cDNA bands which were specifically decreased in the Bmut-b cells in the absence of tetracycline. D, 10 μg of total RNAs of the B-2 and Bmut-b cells were separated on a 1% agarose-formaldehyde gel and analyzed for expression of Egr1 and Mts1 genes by the Northern blot method.
pression of the α2(1) collagen gene which promoter contained a CBF-binding site. This result is consistent with previous studies which showed that mutation of the CBF-binding site in the collagen promoter decreased in vivo transcription activity of the promoter in a transient transfection experiment in fibroblast cells (26). This result indicated that expression of the α2(1) collagen gene is directly regulated by CBF in fibroblasts. Our results also indicated that the inhibition of CBF binding decreased expression of both the E2F1 and topo IIα genes that are activated at different stages of the cell cycle. The promoters of the mouse E2F1 and topo IIα genes consist of multiple CBF-binding sites which are also conserved in the corresponding human promoters (37–39), suggesting that these genes are also directly regulated by CBF. Recent studies of the E2F1 promoter showed that the transcriptional activity of this promoter, like the expression of the endogenous E2F1, is stimulated strongly at the G1/S phase of the cell cycle (38). The E2F1 promoter consists of multiple DNA elements, including binding sites for E2F, SP1, and CBF transcription factors, that regulate repression of the promoter in G0 and activation in S phase. These studies revealed that mutation of the E2F1 site increased the promoter activity in G0, but had little effect in S phase; in contrast, mutation of the CBF-binding site reduced promoter activity only in S phase. These results are consistent with our present observation that the inhibition of CBF binding decreased the activated expression of E2F1 at 15 h after serum stimulation. This indicated that CBF participated in the transcription activation of the E2F1 gene during the period when this gene was stimulated by the growth signal. Since the CBF binding is not regulated during cell growth, it is possible that the CBF-mediated activation of the E2F1 gene is repressed during G0 phase and that, in S phase, CBF activates this gene when the repression is relieved. Recent studies of the mouse topo IIα promoter demonstrated that transcription of this promoter is low in the G1 phase and is stimulated through S phase to a maximal level at G2/M phase of the cell cycle, similar to the case for endogenous topo IIα gene (37). However, the role of the multiple CBF-binding sites in the promoter activity is not known. The human topo IIα promoter also contains multiple CBF-binding sites that, when deleted, resulted in significant loss of the promoter activity, indicating that the CBF-binding sites are part of an upstream activator sequence (39). We speculate that activation of the mouse topo IIα is regulated during the cell cycle by derepression of the CBF-mediated activation. Recent studies showed that transcription activation of several cell cycle-regulated promoters, including the promoters of the cyclin A, Cdc2, and CDC25C genes, appeared to be mediated by a derepression mechanism (40). These promoters consisted of a cell cycle-regulated repressor element and an upstream activator sequence element which mediate repression and activation of the promoter, respectively. The CBF-binding site is found in the upstream activator sequence of these cell cycle-regulated genes, suggesting that inhibition of CBF binding possibly decreased expression of these genes similar to the E2F1 and topo IIα genes.

The inhibition of CBF binding results in a modest decrease of expression of both the E2F1 and topo IIα genes compared with that of the α2(1) collagen gene. The modest decrease of E2F1 and topo IIα expression is somewhat consistent with the observation that the inhibition of CBF binding only delayed but did not completely block the progression of S phase. It is possible that the decreased expression of the α2(1) collagen gene may partly contribute to the retardation of cell growth. The α2(1) collagen is a subunit of the type I collagen which is part of the extracellular matrix protein. Recent studies showed that the extracellular matrix protein and the mitogens together regulate induction of cyclin D and cyclin A in mouse and human fibroblasts, and play an important role in the anchorage-dependent cell cycle progression (43). However, a specific role of the type I collagen in the cell growth process is not known. Although the collagen promoter contains a single CBF-binding site, the inhibition of CBF binding reduced the expression of the collagen gene more than that of the E2F1 and topo IIα genes which promoters contain multiple CBF-binding sites. This indicates that the in vivo transcription of these promoters containing CBF-binding sites are not equally dependent on CBF activity. It seems likely that in case of E2F1 and topo IIα, the contribution of CBF in the in vivo transcription is minor and possibly regulated by other transcription factors which bind to these promoters.

Our differential display experiments indicated that the inhibition of CBF binding did not result in a significant change in expression of many cellular genes. This experiment, however, identified two genes, Egr1 and Mts1 whose expression is specifically decreased by the inhibition of CBF binding. Analysis of the mouse Egr1 and Mts1 promoters revealed that none of the promoters contain consensus CBF-binding site in the proximal promoter region (31, 44), thus indicating that these genes are not directly regulated by CBF. However, it is possible that the CBF-binding site in these promoters may be located in the distal promoter regions which are not yet identified. Altogether our results showed that the inhibition of CBF binding decreased expression of multiple cellular genes, which is consistent with the previous studies that showed that CBF binding to the various promoters is required for optimal transcription activity of the promoters (4). Since expression of none of the genes except the collagen gene are decreased at high levels, it is possible that the inhibition of CBF binding could decrease a smaller amount of expression of many other genes which are required for cell proliferation, and such a smaller decrease of multiple genes could result in significant reduction of the rate of cell growth. However, we cannot rule out a possibility that the inhibition of CBF binding could decrease significantly expression of specific gene(s) which are required for the progression of fibroblast growth. Further studies are needed to test all these possibilities.

The function of CBF homologues in yeast was studied extensively which showed these genes were specifically required for expression of several nuclear genes involved in mitochondrial function (9, 10). Our present results provide direct evidence for the first time that CBF is an important regulator for the normal growth of mouse fibroblast cells, and that it is required for expression of various mouse genes. However, it is possible that CBF may also be required for expression of nuclear genes involved in mitochondrial function in mouse cells, and that the inhibition of CBF binding in fibroblast could disrupt the mitochondrial function, and thus cannot produce adequate energy, which could be an important cause for the retardation of fibroblast growth.

The function of CBF homologues in other eukaryotes was only revealed recently. For example, disruption of the CBF-C homologue aab1 in Neurospora crassa results in very slow growth and altered morphology, thus indicating pleiotropic effects on growth and development (45); and in the higher plant, Arabidopsis thaliana, the CBF-A homologue LEC1 functions at an early developmental stage and activates transcription of genes required for both embryo morphogenesis and cellular differentiation (46). Altogether these results indicated that the function of CBF has diverged during evolution and that this transcription factor likely plays an important role in growth and development of higher eukaryotes.
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