Three Different Polypeptides Are Necessary for DNA Binding of the Mammalian Heteromeric CCAAT Binding Factor

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Full-length cDNA clones for the CBF-A and CBF-B subunits of the CCAAT binding mammalian heteromeric transcription factor (CBF) have previously been isolated from both rat and mouse. Whereas recombinant CBF-B binds to DNA after complementation with a highly purified CBF-A fraction, recombinant CBF-A was unable to bind to DNA after complementation with either purified CBF-B or recombinant CBF-B. However, when recombinant CBF-A, synthesized as a fusion protein with glutathione S-transferase was denatured together with a highly purified fraction containing CBF-A in the presence of 5.5 M guanidine hydrochloride and subsequently renatured, the recombinant CBF-A bound to DNA after complementation with CBF-B. This binding of recombinant CBF-A could not be detected if recombinant CBF-B was not mixed during the denaturation-renaturation process together with the purified fraction containing the 32-kDa CBF-A. Using a Southwestern blot we demonstrated that a polypeptide of approximately 40 kDa, present in the purified CBF-A fraction, bound to DNA after complementation with both recombinant CBF-A and CBF-B. After fractionation of the purified CBF-A preparation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a species of approximately 40 kDa was eluted from the gel and shown to have DNA binding activity after complementation with both recombinant CBF-A and CBF-B. Our results indicate that a third polypeptide, designated CBF-C, forms a tight complex with CBF-A. Together with CBF-A and CBF-B, CBF-C is required for the DNA binding activity of CBF.

The CCAAT binding factor (CBF) is a rat heteromeric DNA-binding transcription activator which binds to specific CCAAT motifs in a number of eukaryotic promoters including those of the α1(1) and α2(1) collagen, albumin, major histocompatibility complex class II, and other genes (5–9). Based on the fact that two distinct chromatographic fractions were needed for DNA binding, we postulated the existence of two different subunits, CBF-A which binds to anionic exchange resins (such as Mono Q) and CBF-B which binds to cationic exchange resins (such as Mono S) (5). Both subunits were purified to apparent homogeneity, and cDNA clones of both subunits were isolated from both rat and mouse (10–12). A portion of the amino acid sequences of each subunit is evolutionarily conserved showing a high degree of sequence identity with segments of the yeast HAP3 and HAP2 polypeptides, respectively. In yeast, the products of the HAP3 and HAP2 genes are necessary for the expression of genes coding for components of the electron transport chain and thus regulate the respiratory function of this organism (14–16). HAP2 and HAP3 are interchangeable with components of the CCAAT-binding protein, C1P1, which is the human homologue of CBF (17). Indeed, the cDNA clone of the human HAP2 homologue has been isolated by functional complementation in a yeast strain bearing a HAP2 mutation (18). The amino acid sequence of the human HAP2 homologue is very similar to that of rat CBF-B. Unlike other heteromeric transcription factors, the sequences of the subunits of CBF do not show any homology with leucine zippers or helix-loop-helix motifs suggesting the existence of a different protein dimerization interface in the CBF protein.

In order to better analyze CBF-A activity, both CBF-A and CBF-B subunits were expressed in *Escherichia coli* as fusion proteins with glutathione S-transferase. Here we report that a third polypeptide of approximately 40 kDa, designated CBF-C, is tightly associated with CBF-A and is necessary to reconstitute the DNA binding activity of CBF using the recombinant forms of CBF-A and CBF-B.

**MATERIALS AND METHODS**

Expression of CBF-A and CBF-B in *E. coli*—Both CBF-A and CBF-B were expressed in *E. coli* as fusion proteins with glutathione S-transferase (19) using the bacterial expression vector, pgEX-2T (Pharmacia LKB Biotechnology Inc.). CBF-A cDNA was fused in frame with glutathione S-transferase into the BamHI and EcoRI sites of the vector. Both BamHI and EcoRI sites were generated into the CBF-A cDNA just preceding the third amino acid codon and following the stop codon, respectively. CBF-B was introduced into the EcoRI site of this plasmid in frame with glutathione S-transferase. EcoRI sites in CBF-B cDNA were generated preceding the third amino acid

The abbreviations used are: CBF, CCAAT binding factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTTF, dithiotreitol; HEPPS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GST, glutathione S-transferase.
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codon and after the stop codon. In each case these modifications were
performed by polymerase chain reactions. The sequence of the ampli-
ified cDNAs were verified by DNA sequencing. Plasmids were
transformed into E. coli strain HB101. The synthesis of fusion pro-
teins was induced by adding 1 mM isopropyl-β-D-thiogalactopyrano-
side for 2 h at the midlog phase of bacterial growth. After bacteria
(500 ml) were pelleted by centrifugation at 3000 rpm for 1 h at 4 °C.
DNA binding was performed by gel shift assays as described previously (10) using a 42 nucleotide double-stranded oligonucleotide containing the sequences of the mouse e2(1) collagen promoter between -105 and -64. CBF-A and CBF-B were purified from rat liver nuclear extracts by a series of chromatographic steps as described earlier (10, 12). The fraction containing CBF-A activity obtained after Mono Q (Pharmacia) chromatography was used as purified CBF-A, and the fraction containing CBF-B activity obtained after Mono S (Pharmacia) chromatography was used as purified CBF-B.

Denaturation and Renaturation of Purified CBF-A and CBF-B Activity from Rat Liver—DNA binding was performed by gel shift assays as described previously (10) using a 42 nucleotide double-stranded oligonucleotide containing the sequences of the mouse e2(1) collagen promoter between -105 and -64. CBF-A and CBF-B were purified from rat liver nuclear extracts by a series of chromatographic steps as described earlier (10, 12). The fraction containing CBF-A activity obtained after Mono Q (Pharmacia) chromatography was used as purified CBF-A, and the fraction containing CBF-B activity obtained after Mono S (Pharmacia) chromatography was used as purified CBF-B.

Renaturation of CBF-C Activity—Purified CBF-A was fractionated in a 10% SDS-PAGE. Nine different slices of 2-3 mm were used for elution from the gel in a buffer containing 0.1% SDS, 50 mM Tris, pH 7.9, 0.2 mM NaCl, 0.1 mM EDTA, 5 mM DTT, and 0.1 mg/ml of bovine serum albumin at 25°C for 12 h. Molecular weights were determined according to the mobility of prestained molecular weight markers (Bethesda Research Laboratories). Proteins were precipitated by 80% acetone and washed with 80% acetone and dried. Proteins were resuspended in 2 X DNA-binding buffer and assayed for DNA binding activity.

Southwestern Blot—Protein fractions were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. After transfer the membranes were blocked with 5% nonfat milk in DNA-binding buffer and washed with 0.25% nonfat milk in the same buffer for 30 min at 4°C. Membranes were then incubated for 2 h at 4°C with 25 ng/ml of recombinant CBF-A, 25 ng/ml of recombinant CBF-B, 100 fmol/ml of "P-labeled (4000 cpm/fmol) double-stranded oligonucleotide containing the CBF-binding site of the chicken beta-globin promoter, poly(dI-C)/poly(dI-C), and 0.25% nonfat milk in DNA-binding buffer. The membranes were washed four times with 0.25% nonfat milk in DNA-binding buffer at room temperature to decrease nonspecific binding. The DNA-bound proteins present on the membranes were detected by autoradiography.

RESULTS

Recombinant CBF-B when expressed in vitro from its cDNA, binds specifically to DNA sequences containing a CCAAT motif after complementation with a chromatically purified native CBF-A fraction (10). The binding properties of the in vitro synthesized CBF-B are identical to those of the chromatically purified native CBF-B in that both CBF-B and CBF-A are required for DNA binding. In contrast, when CBF-A is synthesized in vitro from its cDNA it cannot form a complex with either chromatically purified native CBF-B or with recombinant CBF-B that binds to DNA (12). Peptide antibodies based on the amino acid sequence of the non-conserved COOH-terminal portion encoded by this CBF-A cDNA form a specific higher molecular weight complex with the CBF-DNA complex. This indicates that an amino acid sequence derived from our CBF-A cDNA clone is indeed present in the CBF-DNA complex (13). The possibility that some post-translational modifications, which would not occur in the in vitro expression system would be necessary for the DNA binding activity of CBF, is an unlikely explanation. Indeed, recombinant CBF-A protein, despite being abundantly expressed in a baculovirus system, showed no CBF DNA binding activity after complementation with CBF-B above the activity present in extracts of uninfected insect cells or in extracts of cells infected with wild type baculovirus (data not shown).

The active fraction of the Mono Q chromography purified CBF-A contained a species consisting of a 32-32.5-kDa doublet which showed CBF-A activity after elution and renaturation from SDS-PAGE (12). Amino acid sequences obtained from this 32-32.5-kDa CBF-A species were used in generating the CBF-A cDNA isolated from a rat liver cDNA library. The partial amino acid sequences of all six tryptic peptides derived from the active CBF-A species for which an amino acid sequence was determined, were present in the amino acid sequence deduced from the CBF-A cDNA. Although these results suggested that the 32-32.5-kDa doublet corresponded to a single species, we could not rigorously exclude the presence in the active fraction of another component that would be needed for CBF activity. To test this hypothesis the following experiments were undertaken.

Expression of CBF-A and CBF-B cDNAs in E. coli—Both the CBF-A and CBF-B cDNA were expressed in E. coli by using an expression vector in which the coding sequences of these cDNAs were fused in frame with the glutathione S-transferase (GST) gene (Fig. 1A). This vector system has several advantages. The fusion proteins can be easily purified from crude E. coli extracts by using a glutathione-agarose affinity column (19). As GST adds 26 kDa to the molecular mass of either CBF-A or CBF-B, one could expect the mobility of the DNA-protein complex containing either CBF-A-GST or CBF-B-GST to be slower than that of the complex containing the endogenous CBF subunits in a gel-mobility shift assay. This would allow us, for instance, to distinguish between complexes containing an active CBF-A and CBF-A-GST. Finally, by cleaving at the junction of the fusion, thrombin should restore the molecular weight of the subunits of CBF.

The fused recombinant proteins, expressed in E. coli, were fractionated on SDS-PAGE after purification by glutathione-agarose affinity chromatography (Fig. 1B). The apparent molecular masses of the fusion proteins which constitute the major species in SDS-PAGE, are 66 and 52 kDa for CBF-B and CBF-A, respectively (lanes 2 and 4). When the fusion
proteins were cleaved by thrombin several bands appeared (lanes 3 and 5). A major species corresponded to the molecular weight of native CBF-A (lane 5). The cleavage species of CBF-B-GST migrates somewhat faster than expected for the migration of native CBF-A, suggesting the possibility that thrombin cleaves within the NH2-terminal portion of the fusion proteins. A thrombin cleavage site is present at the junction of the fusion as shown by an arrow. SDS-PAGE analysis of the purified recombinant CBF-A and CBF-B. The purified recombinant proteins (5 µg of each) obtained after glutathione-agarose affinity chromatography were treated with thrombin in a buffer containing 150 mM NaCl, 2.5 mM CaCl2, and 100 ng of human thrombin (Sigma) for 30 min at room temperature. Both the uncleaved and the thrombin-cleaved proteins were fractionated by 10% SDS-PAGE followed by Coomassie Blue staining. Molecular weight prestained marker proteins were used as standards. Lane 1, standards; lane 2, CBF-B-GST; lane 3, thrombin-cleaved CBF-B-GST; lane 4, CBF-A-GST; lane 5, thrombin-cleaved CBF-A-GST. The arrow indicates the position of GST after thrombin cleaved in lanes 3 and 5. Dots correspond to the CBF-B portion of thrombin-cleaved CBF-B-GST (lane 3) and to the CBF-A portion of thrombin-cleaved CBF-A-GST (lane 5). C, DNA binding of the purified CBF-A-GST and CBF-B-GST. The uncleaved and thrombin-cleaved recombinant proteins (500 ng of each) were incubated with 10 fmol of radiolabeled double-stranded oligonucleotide containing the CBF-binding site of the mouse u2(1) collagen promoter and 1 µg of poly[d(I-C)] in 10 µl of DNA-binding buffer. Either the purified CBF-A fraction or the purified CBF-B fraction (0.5 µg of each) were added to the assay mixture to complement either CBF-B-GST or CBF-A-GST, respectively. Reactions were fractionated in a 5% polyacrylamide gel in 0.5 × 89 mM Tris, 89 mM boric acid, 2 mM EDTA. The designation of the different proteins in this figure and the subsequent figures is as follows. CBF-A (Mono Q) is rat liver CBF-A purified as previously reported (12) including Mono Q chromatography; CBF-B (Mono S) is rat liver CBF-B purified according to the previously reported purification scheme including Mono S chromatography (10). CBF-A-GST is purified recombinant CBF-A from E. coli as a fusion with GST, and CBF-B-GST is purified recombinant CBF-B from E. coli. Thrombin-cleaved proteins are designated by the suffix T within parentheses.

Fig. 1. DNA binding of the recombinant CBF-A and the recombinant CBF-B. A, structure of the recombinant CBF-A and the recombinant CBF-B expressed in E. coli. Both CBF-A and CBF-B were expressed in E. coli as fusion proteins with a 26-kDa glutathione S-transferase which forms the NH2-terminal portion of the fusion proteins. A thrombin cleavage site is present at the junction of the fusion as shown by an arrow. B, SDS-PAGE analysis of the purified recombinant CBF-A and CBF-B. The purified recombinant proteins (5 µg of each) obtained after glutathione-agarose affinity chromatography were treated with thrombin in a buffer containing 150 mM NaCl, 2.5 mM CaCl2, and 100 ng of human thrombin (Sigma) for 30 min at room temperature. Both the uncleaved and the thrombin-cleaved proteins were fractionated by 10% SDS-PAGE followed by Coomassie Blue staining. Molecular weight prestained marker proteins were used as standards. Lane 1, standards; lane 2, CBF-B-GST; lane 3, thrombin-cleaved CBF-B-GST; lane 4, CBF-A-GST; lane 5, thrombin-cleaved CBF-A-GST. The arrow indicates the position of GST after thrombin cleaved in lanes 3 and 5. Dots correspond to the CBF-B portion of thrombin-cleaved CBF-B-GST (lane 3) and to the CBF-A portion of thrombin-cleaved CBF-A-GST (lane 5). C, DNA binding of the purified CBF-A-GST and CBF-B-GST. The uncleaved and thrombin-cleaved recombinant proteins (500 ng of each) were incubated with 10 fmol of radiolabeled double-stranded oligonucleotide containing the CBF-binding site of the mouse u2(1) collagen promoter and 1 µg of poly[d(I-C)] in 10 µl of DNA-binding buffer. Either the purified CBF-A fraction or the purified CBF-B fraction (0.5 µg of each) were added to the assay mixture to complement either CBF-B-GST or CBF-A-GST, respectively. Reactions were fractionated in a 5% polyacrylamide gel in 0.5 × 89 mM Tris, 89 mM boric acid, 2 mM EDTA. The designation of the different proteins in this figure and the subsequent figures is as follows. CBF-A (Mono Q) is rat liver CBF-A purified as previously reported (12) including Mono Q chromatography; CBF-B (Mono S) is rat liver CBF-B purified according to the previously reported purification scheme including Mono S chromatography (10). CBF-A-GST is purified recombinant CBF-A from E. coli as a fusion with GST, and CBF-B-GST is purified recombinant CBF-B from E. coli. Thrombin-cleaved proteins are designated by the suffix T within parentheses.

In short, the thrombin-induced mobility change of the DNA-protein complex must be due to a decrease in molecular weight of the CBF-B-GST polypeptide. This experiment strongly suggested that the GST addition did not prevent the recombinant CBF-B-GST from participating in the DNA-protein complex. In contrast, CBF-A-GST, whether uncleaved or cleaved by thrombin, had no DNA binding activity after complementation with purified CBF-B (lanes 6 and 7). Thus, irrespective of the expression system used (E. coli, in vitro transcription translation, baculovirus) recombinant CBF-B showed activity similar to that of native CBF-B whereas recombinant CBF-A had no activity.

The Purified CBF-A Fraction Consists of a Complex Containing the CBF-A Polypeptide and an Another Component, CBF-C—If the highly purified chromatographic fraction containing the 32–32.5-kDa CBF-A species also contained another component which would be needed together with the CBF-A polypeptide, to form a protein-DNA complex after complementation with CBF-B, one may expect that the recombinant CBF-A-GST would associate with the same component in the formation of a DNA-protein complex. To test this hypothesis, a mixture of CBF-A-GST and the purified CBF-A fraction (in a molar ratio of approximately 250:1) was assayed for DNA binding activity (Fig. 2A). Based on the ability of CBF-B-GST to upshift the complex (Fig. 1C, lane...
Fig. 2. DNA binding of the mixture of CBF-A-GST and the purified fraction containing CBF-A denatured and renatured by guanidine hydrochloride or by heat treatment. A, DNA binding of the mixture of the purified CBF-A fraction, purified CBF-B and CBF-A-GST. The CBF-A-GST (600 ng) was added in the DNA-binding reaction assay containing the purified fraction containing CBF-A (0.5 ng) and the purified CBF-B fraction (0.5 ng). DNA binding assays were performed by gel shift assay as described in Fig. 1C. B, DNA binding of the mixture of CBF-A-GST and the purified fraction containing CBF-A denatured and renatured by guanidine hydrochloride treatment. The renatured proteins (18 μl) were incubated with 10 fmol of the radiolabeled double-stranded oligonucleotide in DNA-binding buffer, with or without purified CBF-B (0.5 ng). DNA binding assays of the untreated proteins were performed as described in Fig. 1C. The guanidine hydrochloride-denatured and -renatured proteins are marked by the prefix GD. When a mixture of proteins were denatured together by guanidine hydrochloride these proteins are indicated within a bracket following the prefix GD. C, DNA binding of the renatured mixture of purified CBF-A and the recombinant CBF-A cleaved by thrombin. The denatured and renatured protein mixture of the purified fraction containing CBF-A plus recombinant CBF-A (18 μl), (as was used in the experiment shown in B, lane 2) was incubated with 100 ng of human thrombin at room temperature for 30 min. The DNA binding activity of the thrombin-cleaved protein mixture was assayed by addition of 10 fmol of radiolabeled oligonucleotide and 0.5 ng of purified CBF-B (lane 2). For the control experiment, the renatured protein mixture (18 μl) was incubated at room temperature for 30 min without thrombin, and the DNA binding activity was assayed as before (lane 1). D, DNA binding of the heat-treated mixture containing the purified CBF-A fraction and recombinant CBF-A. The mixture of the purified fraction containing CBF-A (0.5 ng) and the recombinant CBF-A (500 ng) was incubated at 95 °C for 5 min in 9 μl of DNA-binding buffer. The DNA binding activity of the heat-treated proteins were assayed after addition of 10 fmol of radiolabeled double-stranded oligonucleotide containing the CBF-binding site, 1 μg of poly(dI-C)/poly(dI-C), and 0.5 ng of purified CBF-B. The purified CBF-B was omitted when the heat-treated mixture contained 0.5 ng of purified CBF-B. The heat-treated proteins were marked by the prefix HT. When a mixture of proteins was heat treated, they are indicated within a bracket following the prefix HT.

2), one could expect that if CBF-A-GST was present in the DNA-protein complex, the mobility of that complex would be slower than that containing the endogenous native CBF-A. No such slower mobility complex was observed when both the purified CBF-A fraction and CBF-A-GST were assayed together in the DNA binding reaction (lane 2). The only change was the disappearance of a minor faster mobility complex, designated α in Fig. 2A. This minor complex was previously interpreted as representing a proteolytic segment of CBF-A which could still associate with CBF-B to form a faster mobility DNA-protein complex (5). The significance of the disappearance of this minor band is not well understood.

The experiment shown in Fig. 2A did not rule out the possibility that in the purified fraction containing the 32-32.5-kDa CBF-A a component is present that is complexed with the endogenous CBF-A polypeptide. If the dissociation constant of this complex was low, little exchange would occur between endogenous native CBF-A and recombinant CBF-A polypeptides. To facilitate such an exchange, CBF-A-GST and the purified CBF-A fraction were denatured together (molar ratio of CBF-A-GST/CFB-A of 250:1) in presence of 5.5 M guanidine hydrochloride and the proteins renatured after dilution of the guanidine hydrochloride to 0.22 M. The renatured protein mixture was then tested for DNA binding after complementation with native CBF-B (Fig. 2B). The DNA binding activity of the renatured mixture containing CBF-A-GST and the purified CBF-A fraction was greater than that of the guanidine treated and renatured purified CBF-A fraction alone (compare lanes 2 and 3). In addition, the mobilities of the major DNA-protein complexes formed with the guanidine-treated and renatured mixture of CBF-A-GST plus the purified CBF-A fraction (c and d) are slower than those of the DNA-protein complex formed with the guanidine-treated and renatured purified CBF-A fraction alone. This change of mobility of the DNA-protein complexes did not occur as a result of guanidine hydrochloride treatment because there was no change in mobility of the DNA-protein complexes formed after guanidine hydrochloride treatment of the purified CBF-A fraction (compare lanes 1 and 3). Guanidine treatment and renaturation of the CBF-A fraction itself also decreased its activity (compare lanes 1 and 3).

When a 250-fold molar excess of CBF-A-GST was added to the purified CBF-A fraction, after this CBF-A fraction by itself had been treated with guanidine and renatured, the DNA-protein complex formed with this mixture had a similar intensity and a similar mobility as that formed with the renatured purified CBF-A fraction alone (lanes 3 and 4). Furthermore, in another experiment, CBF-A-GST and the purified CBF-A fraction were denatured and renatured separately and then mixed together in the DNA binding assay. Both the intensity and the mobility of the DNA-protein complexes formed with this mixture were very similar to that of the DNA-protein complex formed with the renatured purified CBF-A alone (data not shown). Thus, it appeared that both the stimulation of DNA binding and the change in mobility of the DNA-protein complexes formed with the
mixture of CBF-A-GST and the purified fraction containing CBF-A occurred only when the constituents of this mixture were denatured and renatured together. We interpret these results as indicating that CBF-A-GST participated in the formation of the slower mobility DNA-protein complexes together with a component, designated here as CBF-C, which is present in the purified CBF-A fraction as a complex with the endogenous CBF-A polypeptide. This CBF-A-CBF-C complex is only dissociated with high concentrations of guanidine hydrochloride. No exchange of this CBF-C component occurred in aqueous buffer between the CBF-A polypeptide and the CBF-A-GST polypeptide even when CBF-A-GST was present in a 250-fold molar excess over CBF-A. We believe that CBF-C became associated mostly with the CBF-A-GST polypeptide in the reaction shown in Fig. 2B, lane 2, because CBF-A-GST was present in molar excess over the native CBF-A polypeptide during the dissociation and reassociation process.

To verify that the slower mobility DNA-protein complexes contained the CBF-A-GST polypeptide, the renatured mixture of CBF-A-GST and the purified CBF-A fraction was treated with thrombin to remove the GST portion from the CBF-A-GST polypeptide (Fig. 1B). This increases the mobility of the DNA-protein complex which now becomes very similar to that of the DNA-protein complex formed with the purified CBF-A fraction (Fig. 2C, lane 2). We interpret this change in mobility of the DNA-protein complexes following thrombin treatment as we interpreted the thrombin-induced change in mobility of the complex containing CBF-B-GST (Fig. 1C); that it is due to a decrease in molecular weight of the CBF-A-GST polypeptide. This result strongly suggests that the CBF-A-GST polypeptide is present in the slower mobility DNA-protein complexes.

The DNA binding activity of the purified CBF-A fraction is heat stable. To test whether CBF-C could be exchanged from its complex with native CBF-A to associate with CBF-A-GST at high temperature, the mixture containing the purified CBF-A fraction and the CBF-A-GST polypeptide was heated to 95 °C for 5 min and then assayed for DNA binding after complementation with CBF-B (Fig. 2D). Heat treatment of the purified CBF-A fraction resulted in the loss of the faster mobility minor DNA-protein complex but little change occurred in the slower mobility major DNA-protein complex (lane 1). The mobilities of the DNA-protein complexes formed either with the heat-treated purified CBF-A fraction or with the heat-treated mixture containing both purified CBF-A and CBF-A-GST were identical (lanes 1 and 2). This suggests CBF-C cannot be exchanged between the native CBF-A polypeptide and CBF-A-GST as a result of the 95 °C treatment. Thus, it appears that the CBF-A-CBF-C complex is not dissociable by heat although it is by 5.5 M guanidine hydrochloride.

The CBF-C Component Is a Polypeptide of Approximately 40 kDa—Having shown the existence of a third component for DNA binding of CBF, we wished to identify CBF-C using a Southwestern blot experiment. The purified CBF-A preparation was fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and then incubated with CBF-A-GST, CBF-B-GST, and a 32P labeled double-stranded oligonucleotide containing the CBF-binding site. A 40-kDa polypeptide present in the purified CBF-A fraction bound DNA only in the presence of both CBF-A-GST and CBF-B-GST (Fig. 3A, lane 14) but not in presence of either DNA alone or DNA and CBF-A-GST (lanes 2 and 6). The 40-kDa species formed a very weak complex in the presence of DNA and CBF-B-GST barely detectable on the autoradiogram (lane 10). When the DNA contained a point mutation in the CBF-binding site, which abolishes CBF binding, no binding occurred to the 40-kDa species when both CBF-A-GST and CBF-B-GST were present (data not shown) indicating that an intact CBF-DNA-binding site is required for binding to the 40-kDa species on the Southwestern blot. The purified CBF-A preparation used in this experiment which is shown in Fig. 3B, contains, as reported previously four different major polypeptides (1', 2', and 3') of 45, 40, 32.5 and 32 kDa. This experiment strongly suggests that it is the 40-kDa polypeptide which formed a specific complex with DNA in presence of the CBF-A-GST and CBF-B-GST polypeptides and which is, therefore, the CBF-C component.

Fig. 3 also shows Southwestern results obtained with the same reagents using a less purified chromatographic fraction containing CBF activity. This fraction was obtained after passing crude liver nuclear extracts over a DNA affinity column containing the CBF-binding site. This affinity purified fraction was about 200-fold purified from crude nuclear extracts compared to the 30,000-fold purification of the CBF-A fraction (12). Several polypeptides present in this fraction bound to DNA in absence of CBF-A-GST and CBF-B-GST (lane 4). The 40-kDa polypeptide which could form a complex with DNA in the presence of both CBF-A-GST and CBF-B-GST with the purified fraction was not easily discernible in this first affinity purified fraction due to the presence of nonspecific complexes which have similar molecular weights (lane 16). Moreover, these same nonspecific complexes were also seen when a mutant DNA probe was used.

The highly purified Mono Q-purified fraction containing CBF-A was tested in a Western blot using as an antibody against CBF-A (13). The antibody only bound to the 32-32.5-kDa species and not to the other two polypeptides (data not shown). This suggest that the 40-kDa species is very likely not an isoform of CBF-A, a fact that was already evident from the molecular mass of CBF-A deduced from the cDNA clones. To further confirm that a species of approximately 40 kDa that is different from both CBF-A and CBF-B is needed for DNA binding of CBF, the purified CBF-A preparation was fractionated by SDS-PAGE. The gel was sliced in fractions of different molecular weights and the eluted species assayed for DNA binding. Fig. 4 shows that the fraction of 43 to 40 kDa has DNA binding activity after complementation with both CBF-A-GST and CBF-B-GST (lane 13). This fraction showed no DNA binding activity after complementation with either CBF-B-GST alone (lane 4) or CBF-A-GST alone (not shown). This experiment demonstrates that three different components, CBF-A, CBF-B, and CBF-C, are needed for formation of the CBF-DNA complex. The only other fraction which showed activity in the presence of CBF-B-GST and CBF-A-GST has an approximate molecular mass of 19-21 kDa. We believe that this fraction probably represents a degradation product of CBF-C.

DISCUSSION

Our results indicate that recombinant CBF-A is fully active but needs the presence of an additional polypeptide, identified as a species of approximately 40 kDa, designated CBF-C. Based on our experiments we propose that CBF-A forms a very stable non-covalent complex with CBF-C. This complex together with CBF-B forms a heteromeric CBF protein which binds to DNA. Whereas CBF-B and the CBF-A-CBF-C complex are easily dissociable from each other by ion-exchange
protein pellets resuspended in 2 standards are shown in cleotide, CBF-A. GST and CBF-Be GST. After elution, proteins were precipitated by acetone and the dried approximate molecular masses: that a SDS-PAGE fraction containing the 32-32.5-kDa double-stranded radiolabeled oligonucleotide containing the CBF-B.GST; and is needed for transcription activation by this heteromeric transcription factor, recent studies from the same laboratory have demonstrated that a fusion protein containing HAP2 fused to the acidic activation domain of GAL4 could at least partially overcome the growth deficiency of a HAP4 deletion mutant but could not complement a HAP3 deletion mutant (21). This observation suggested a model in which site-specific DNA binding was accomplished by both the HAP2 and HAP3 genes are the homologues of mammalian CBF-B and CBF-A, respectively. Although previous studies by Forsberg and Guarente (20) had suggested that the product of the HAP4 gene is present in the DNA-binding complex and is needed for transcription activation by this heteromeric transcription factor, recent studies from the same laboratory have demonstrated that a fusion protein containing HAP2 fused to the acidic activation domain of GAL4 could at least partially overcome the growth deficiency of a HAP4 deletion mutant but could not complement a HAP3 deletion mutant (21). This observation suggested a model in which site-specific DNA binding was accomplished by both the HAP2 and HAP3 genes are the homologues of mammalian CBF-B and CBF-A, respectively. Although previous studies by Forsberg and Guarente (20) had suggested that the product of the HAP4 gene is present in the DNA-binding complex and is needed for transcription activation by this heteromeric transcription factor, recent studies from the same laboratory have demonstrated that a fusion protein containing HAP2 fused to the acidic activation domain of GAL4 could at least partially overcome the growth deficiency of a HAP4 deletion mutant but could not complement a HAP3 deletion mutant (21). This observation suggested a model in which site-specific DNA binding was accomplished by both the HAP2 and HAP3 genes are the homologues of mammalian CBF-B and CBF-A, respectively. Although previous studies by Forsberg and Guarente (20) had suggested that the product of the HAP4 gene is present in the DNA-binding complex and is needed for transcription activation by this heteromeric transcription factor, recent studies from the same laboratory have demonstrated that a fusion protein containing HAP2 fused to the acidic activation domain of GAL4 could at least partially overcome the growth deficiency of a HAP4 deletion mutant but could not complement a HAP3 deletion mutant (21). This observation suggested a model in which site-specific DNA binding was accomplished by both the HAP2 and HAP3 subunits, whereas the HAP4 subunit associated with this complex to provide a transcriptional activation domain. The requirement of HAP4 for transcription activation could be substituted with a GAL4 transcription activation domain present in the HAP2-GAL4 fusion. Our biochemical analysis indicates that the presence of CBF-C is completely required for the participation of CBF-A together with CBF-B in the CBF-DNA complex. These properties of CBF-C favor the notion that CBF-C is different from HAP4.
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