Three Classes of Mutations in the A Subunit of the CCAAT-Binding Factor CBF Delineate Functional Domains Involved in the Three-Step Assembly of the CBF-DNA Complex

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The mammalian CCAAT-binding factor CBF (also called NF-Y or CP1) consists of three subunits, CBF-A, CBF-B, and CBF-C, all of which are required for DNA binding and present in the CBF-DNA complex. In this study we first established the stoichiometries of the CBF subunits, both in the CBF molecule and in the CBF-DNA complex, and showed that one molecule of each subunit is present in the complex. To begin to understand the interactions between the CBF subunits and DNA, we performed a mutational analysis of the CBF-A subunit. This analysis identified three classes of mutations in the segment of CBF-A that is conserved in *Saccharomyces cerevisiae* and mammals. Analysis of the first class of mutants revealed that a major part of the conserved segment was essential for interactions with CBF-C to form a heterodimeric CBF-A/CBF-C complex. The second class of mutants identified a segment of CBF-A that is necessary for interactions between the CBF-A/CBF-C heterodimer and CBF-B to form a CBF heterotrimer. The third class defined a domain of CBF-A involved in binding the CBF heterotrimer to DNA. The second and third classes of mutants acted as dominant negative mutants inhibiting the formation of a complex between the wild-type CBF subunits and DNA. The segment of CBF-A necessary for DNA binding showed sequence homology to a segment of CBF-C. Interestingly, these sequences in CBF-A and CBF-C were also homologous to the sequences in the histone-fold motifs of histones H2B and H2A, respectively, and to the archaeobacterial histone-like protein Hmf-2. We discuss the functional domains of CBF-A and the properties of CBF in light of these sequence homologies and propose that an ancient histone-like motif in two CBF subunits controls the formation of a heterodimer between these subunits and the assembly of a sequence-specific DNA-protein complex.

Many eukaryotic transcription factors bind to specific DNA sequences in promoters either as homodimers or as heterodimers and fall into several classes according to the nature of their protein dimerization interfaces (37). Other heteromeric transcription factors consist of multiple polypeptides, such as the complexes that contain the herpes simplex virus protein VP16 (8, 24), the interferon-stimulated complex (14, 22), and GABP, the GA-binding protein complex (25, 41). However, in these cases one of the polypeptides in each of these complexes has the ability to bind to DNA in the absence of the other proteins usually present in these complexes.

The mammalian CCAAT-binding factor CBF (also called NF-Y and CP1) is an evolutionarily conserved heteromeric DNA-binding protein that binds to CCAAT motifs in the promoters of numerous eukaryotic genes (7, 10, 19, 27, 29, 32). In many of the higher eukaryotic genes, the CCAAT motifs are located between –60 and –100 bp 5' to the start of transcription (6). The promoter sequences around these CCAAT motifs are also highly conserved when specific genes are compared in different species. In contrast to other proteins such as C/EBP and CTF/NF1, which have also been designated CCAAT-bind-

ing proteins, CBF has a stringent requirement for the CCAAT pentanucleotide to bind to DNA (10, 21, 29).

CBF consists of three different subunits, CBF-A, CBF-B, and CBF-C (30). The A and B subunits in rats, mice, and humans show almost 99% amino acid sequence identity (4, 26, 31, 43, 44). Furthermore, part of the amino acid sequence of CBF-A and part of CBF-B show a high degree of sequence identity with segments of the *Saccharomyces cerevisiae* HAP3 and HAP2 proteins, respectively (17, 38). These two polypeptides are subunits of a multimeric yeast CCAAT-binding factor that regulates expression of several nuclear genes essential for mitochondrial function (13, 16, 36).

We recently isolated cDNAs for rat CBF-C, the third subunit of CBF, and with recombinant polypeptides demonstrated that all three subunits of CBF (CBF-A, CBF-B, and CBF-C) are needed for DNA binding and that all three polypeptides are present in the CBF-DNA complex (40). A yeast homolog of CBF-C, designated HAP5, has also been isolated (33). Analysis of the amino acid sequences of the CBF subunits showed no homology with any of the known DNA-binding and dimerization motifs found in other heteromeric DNA-binding proteins. Together, these properties classify CBF as a unique DNA-binding protein.

A previous mutational analysis of CBF-B indicated that the conserved segment of this subunit necessary for DNA binding consists of two domains, a domain for interactions with the other CBF subunits and a DNA-binding domain (28). Similar conclusions were also reached by mutational analysis of the yeast HAP2 polypeptide (35). Mutational data as well as circular dichroism studies of an oligopeptide covering the subunit

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interaction domain of HAP2 were consistent with an α -helical configuration for this domain (48). However, the mechanism by which the other two subunits, CBF-A and CBF-C, participate in the formation of the CBF-DNA complex remains to be determined.

In this study we first determined the subunit stoichiometries of both the CBF molecule and the CBF-DNA complex. To determine how CBF-A interacts with the other two subunits and with DNA, we performed a mutational analysis of this CBF subunit. This analysis first revealed that the segment of CBF-A that is conserved in *S. cerevisiae* and mammalian species is necessary and sufficient for DNA binding. Within this conserved segment we distinguished three different classes of mutants corresponding to three functional interaction domains. Unlike the modular arrangements of domains in many other transcription factors, these interaction domains in CBF-A overlapped each other. The three classes define the role of functional domains of CBF-A in each of the three sequential steps required for the formation of the CBF-DNA complex. Interestingly, the conserved regions of CBF-A and CBF-C have sequence similarities to the histone-fold motifs of histones H2B and H2A, respectively, and to the archaeobacterial histone-like protein Hmf-2.

MATERIALS AND METHODS

In vitro transcription-translation. To improve their translational efficiencies, the subunits of CBF were cloned in the pCITE-2a vector (Novagen) by PCR. The DNA corresponding to the open reading frame of CBF-B was subcloned between the *Nde*I and *Xho*I restriction sites of the pCITE-2a vector. Similarly, full-length CBF-A DNA was cloned between the *Eco*RI and *Xho*I sites of the pCITE-2a vector. The cloning of CBF-C DNA in the pCITE vector has been described previously (40). To express each of the CBF subunits, in vitro transcription and translation were performed with these DNAs by the Novagen single tube protein system according to the manufacturer's protocol. For cross-linking and GST pull-down experiments, proteins were labeled with [³⁵S]methionine during translation.

Cross-linking of CBF subunits. For cross-linking studies, CBF subunits expressed in the reticulocyte lysate were incubated separately or in various combinations with glutaraldehyde (0.02% final concentration) in phosphate-buffered saline. After a 15-min incubation, the cross-linked products were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE). Subsequent to electrophoresis, the gels were fixed, dried, and exposed to autoradiography.

In vitro protein-protein interaction assays. For the glutathione *S*-transferase (GST) pull-down assays, [³⁵S]methionine-labeled CBF-C was incubated separately with either GST, GST–CBF-A, or GST–CBF-A mutants in a buffer containing 25 mM HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol for 20 min. Ten microliters of packed glutathione-agarose resin (Sigma) was added to the reaction mixture in a volume of 100 μ l. After incubating the reaction mixture with the resin for 20 min, the reaction mixture was centrifuged and the resin was washed three times with 500 μ l of incubation buffer. The resin was then boiled in SDS-PAGE loading buffer and run on an SDS–12.5% polyacrylamide gel that was subsequently dried and exposed. The interaction assays with CBF-B were performed in the same manner except that [³⁵S]CBF-B was used and all reaction mixtures also contained 500 ng of GST–CBF-C.

Stoichiometry of CBF. The DNA for the truncated form of CBF-C corresponding to amino acids 1 to 175 was cloned into the *Eco*RI and *Xho*I sites of the pCITE-2b vector (Novagen). The DNA for the truncated form of CBF-A corresponding to amino acids 53 to 160 of CBF-A was cloned in the *Nco*I and *Sal*I sites of the pCITE-2a vector. For truncated CBF-B, the DNA corresponding to residues 251 to 341 was cloned into the *Hind*III and *Xho*I sites in pBluescript-SK plasmid. The full-length and truncated CBF subunits were expressed in the reticulocyte lysate (Novagen) with or without [³⁵S]methionine. Whereas the labeled products were analyzed by SDS-PAGE to verify the molecular masses of the proteins that were synthesized, the unlabeled products were used in gel shift assays. For gel shift assays, full-length CBF-C and truncated CBF-C were assayed with recombinant CBF-A (rCBF-A) and rCBF-B while CBF-A was assayed with rCBF-B and bacterially made CBF-C with a deletion of the carboxyl-terminal 159 residues (CBF-C_{del}). rCBF-A, rCBF-B, and rCBF-C have been described previously (40). The two forms of CBF-B were assayed with a highly purified fraction containing native rat CBF-A and CBF-C subunits. Gel shift assays were performed with a probe containing the sequence of the mouse α 2(I) collagen promoter from positions –64 to –105 (31).

Generation of mutants of CBF-A. The amino- and carboxyl-terminal deletions of the CBF-A polypeptides were generated by using a PCR approach. A *Bam*HI site for the N-terminal deletions and an *Eco*RI site for the C-terminal deletions were created in each of the oligonucleotides that were used to generate specific deletions. Sense and antisense oligonucleotides were used in a PCR with GST–CBF-A DNA as the template, and PCR products were digested with *Bam*HI and *Eco*RI and cloned into the pGEX-2T vector (Pharmacia). Internal deletions and amino acid substitution mutations were generated by a two-step PCR (28). For some double or triple amino acid substitutions, pGEX-2T–CBF-A DNA already containing one mutation in CBF-A was used as the template for the two-step PCR with the oligonucleotides containing the other desired mutation. All mutants were verified by DNA sequencing. Mutants of CBF-A were expressed in *Escherichia coli* as described previously (30).

Yeast two-hybrid assay. All interaction studies were performed with the yeast strain Y190 (a kind gift of S. J. Elledge) with two expression vectors, pAS1, containing a tryptophan marker, and pACT-II, containing a leucine marker (11, 18). The DNA corresponding to residues 44 to 151 of CBF-A was cloned in frame downstream of the GAL4 DNA-binding domain in the pAS1 vector between the *Nco*I and *Bam*HI sites. The DNA corresponding to residues 27 to 123 of CBF-C and residues 253 to 320 of CBF-B was cloned in frame downstream of the GAL4 activation domain at the *Sma*I and *Xho*I sites of the pACT-II vector. DNA containing mutations in CBF-A were introduced in the pAS1 vector by PCR with GST–CBF-A DNA containing mutations as the templates. The reading frames and the sequences of all the constructs were verified by DNA sequencing. Yeast strain Y190 was transformed with the vectors or their recombinant derivatives and selected on plates lacking the appropriate amino acids according to standard protocol (15). Colonies were grown at 30°C for 2 to 5 days, and the β -galactosidase activity was monitored by the colony filter lift method (5). Briefly, after yeast colonies were transferred to a nitrocellulose membrane and lysed with liquid nitrogen, the membranes were soaked in buffer containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) reagent at 30°C. The appearance of blue color was monitored at different time points.

RESULTS

Stoichiometries of the three CBF subunits in the CBF molecule and in the CBF-DNA complex. We showed previously that CBF-A and CBF-C associate with each other to form a CBF-A/CBF-C complex and that CBF-B does not interact with either CBF-A or CBF-C separately but rather associates only with the CBF-A/CBF-C complex to form a ternary CBF protein complex (40). One essential requirement to further understand these interactions is to determine the stoichiometry of each CBF subunit in the ternary complex. We performed cross-linking experiments using full-length CBF subunits synthesized in the reticulocyte lysate system. Two CBF subunits in three different combinations were cotranslated and subsequently cross-linked, whereas in control experiments individual CBF subunits were cross-linked separately. The cross-linked products were analyzed on SDS-polyacrylamide gel, and the results are shown in Fig. 1. A specific cross-linked product was formed only in the reaction in which both CBF-A and CBF-C were cotranslated (Fig. 1, lane 12), demonstrating that CBF-A interacts with CBF-C. No specific cross-linked products were observed in the reactions containing either CBF-A and CBF-B (Fig. 1, lane 8) or CBF-B and CBF-C (lane 10). The apparent molecular mass of the cross-linked CBF-A/CBF-C product of about 70 kDa corresponds to a heterodimer containing one molecule of 32 kDa CBF-A and one molecule of 40 kDa CBF-C. In the reactions of lanes 2, 6, and 12, no homodimers of CBF-A or CBF-C were formed. When the cross-linking reaction mixture contained all three CBF subunits, two major specific cross-linked products were formed, one corresponding to the CBF-A/CBF-C complex and the other to a molecule consisting of one of each subunit on the basis of molecular mass (Fig. 1, lane 14). This experiment indicates that CBF-B interacts with the CBF-A/CBF-C complex to form a ternary CBF-B(CBF-A/CBF-C) complex, providing direct evidence for the formation of a heterotrimeric CBF complex in the absence of DNA binding. These results also support our previous conclusions regarding the pathway of assembly of the heterotrimeric complex (40).

FIG. 1. Cross-linking assays to examine interactions between the CBF subunits. Two microliters of reticulocyte lysate containing either one [35 S]methionine-labeled CBF subunit (lanes 1 to 6) or two cotranslated subunits of CBF (lanes 7 to 14) was used for the cross-linking assay. In lanes 13 and 14, 200 ng of rCBF-B made in *E. coli* was added to the reaction mixture. The high-molecular-mass cross-linked products in lanes 4 and 8 are probably multimeric forms of CBF-B; those in lanes 6 and 10 are probably multimeric forms of CBF-C. The locations of molecular mass standards run on the same gels are indicated on the left.

To determine the stoichiometry of the CBF subunits in the CBF-DNA complex, DNA-binding assays were performed with full-length and a deleted form of each subunit in combination with the other two CBF subunits. A full-length CBF-B and a CBF-B_{del} containing only residues 251 to 341 were synthesized in the rabbit reticulocyte lysate. Previous studies (28) indicated that CBF-B_{del} binds DNA as efficiently as full-length CBF-B when complemented with the native CBF-A and CBF-C subunits (Fig. 2A, lanes 1 and 2). Because the two DNA-protein complexes contain identical CBF-A and CBF-C subunits, any difference in the mobilities of the two complexes would be due to a difference in the molecular mass of CBF-B. When full-length CBF-B and CBF-B_{del} were synthesized together in the rabbit reticulocyte lysate and then assayed for DNA binding, only two DNA-protein complexes were formed, one that corresponded to a complex containing full-length CBF-B and one to a complex containing CBF-B_{del} (Fig. 2A, lane 3). If two molecules of CBF-B were present in the CBF-DNA complex, a third complex with an intermediate level of mobility containing one molecule of full-length CBF-B and one molecule of CBF-B_{del} would have appeared, but none did. This experiment thus indicated that only one molecule of CBF-B is present in the CBF-DNA complex. Similar experiments were performed to determine the stoichiometries of CBF-A and of CBF-C in the CBF-DNA complex (Fig. 2B, lanes 1 to 6). CBF-A_{del} contained only residues 53 to 160 and CBF-C_{del} contained only the 175 N-terminal residues. Preliminary results have shown that the segment of CBF-C containing residues 27 to 123 is necessary and sufficient for DNA binding (39a). In both cases, the mobility of the DNA-protein complex containing a full-length molecule was lower than that of the DNA-protein complex containing the truncated version (Fig. 2B, lanes 1 and 2 for CBF-A and lanes 4 and 5 for CBF-C). The same two DNA-protein complexes were also formed in a DNA-binding reaction containing a mixture of either full-length CBF-A and CBF-A_{del} (Fig. 2B, lane 3) or full-length CBF-C and CBF-C_{del} (lane 6). No additional DNA-protein complex with an intermediate level of mobility was formed in either case. Hence, we conclude that the CBF-DNA complex contains one molecule of each of the three CBF subunits.

Segments of CBF-A that are necessary for DNA binding. To determine which segment of CBF-A was necessary for DNA binding, both amino-terminal and carboxyl-terminal deletions were generated (see Table 1 for a schematic representation of the mutants). Deleted polypeptides were expressed in *E. coli* as fusions with GST, purified from crude *E. coli* extracts by glu-

tathione-affinity chromatography (Fig. 3A, lanes 2 to 6), and tested in DNA-binding reactions (Fig. 3B, lanes 1 to 5). Mutants 53-207 and 53-142 bound to DNA like wild-type CBF-A (Fig. 3B, lanes 1 to 3). In contrast, mutants 63-142 and 53-120 did not form a DNA-protein complex (Fig. 3B, lanes 4 and 5). This experiment indicates that the segment of CBF-A between residues 53 and 142 was necessary for DNA binding. This

FIG. 2. DNA binding of full-length and truncated CBF subunits. Full-length and truncated CBF subunits were synthesized in a reticulocyte lysate as described in Materials and Methods. Two microliters of the reticulocyte lysate was used for each DNA-binding reaction. DNA-protein complexes were separated by electrophoresis for 2 h (A) and 4 h (B), respectively. (A) CBF-B in lanes 1 to 3 was assayed with a preparation containing 5 ng of purified native CBF-A and CBF-C. Lane 1, CBF-B; lane 2, CBF-B_{del}; lane 3, cotranslational products of both full-length CBF-B and CBF-B_{del}. (B) CBF-A in lanes 1 to 3 was assayed with rCBF-B (20 ng) and bacterially made CBF-C_{del} (20 ng) containing the N-terminal 175 amino acids. The CBF-C_{del}, expressed as a GST-fusion product, was separated from the GST moiety with thrombin; this polypeptide was used in this assay to enhance the difference in the levels of mobility of the DNA-protein complex with full-length CBF-A and with truncated CBF-A. Lane 1, CBF-A; lane 2, CBF-A_{del}; lane 3, cotranslational products of both full-length CBF-A and CBF-A_{del}. CBF-C in lanes 4 to 6 was assayed with rCBF-B (20 ng) and rCBF-A (20 ng). Lane 4, CBF-C; lane 5, CBF-C_{del}; lane 6, cotranslational products of both full-length CBF-C and CBF-C_{del}.

TABLE 1. Properties of deletion mutants of CBF-A

Deletion mutants of CBF-A ^a	DNA binding ^b	In vitro interactions ^c	
		CBF-C	CBF-B
1 50 100 150 207			
wt	+	+	+
53-207	+	+	+
53-142	+	+	+
63-142	-	-	-
53-120	-	-	-
Δ51-56	+	+	+
Δ57-62	-	+	+
Δ63-70	-	-	-
Δ71-77	-	-	-
Δ79-85	-	-	-
Δ87-93	-	-	-
Δ94-99	-	-	-
Δ103-108	+	+	+
Δ112-119	-	-	-
Δ122-127	-	-	-
Δ130-135	±	-	-

^a Wild-type (wt) and deletion mutants of CBF-A which were expressed as GST-fusion proteins are shown schematically. The numbers to the left of the amino-terminal and carboxyl-terminal deletions indicate the amino acid residues that were included. The internal deletions are shown with gaps and are indicated to the left with a Δ sign followed by the positions of the amino acid residues that were deleted.

^b DNA binding is indicated as identical to wild-type CBF-A (+), no binding observed (-), or weak binding (±).

^c Interactions with CBF-C and CBF-B are indicated as wild-type interactions (+) or no interactions (-).

segment also showed a high degree of amino acid sequence identity with a part of the yeast HAP3 polypeptide (17, 44).

To analyze this segment of CBF-A in greater detail, a series of in-frame internal deletions were constructed as GST-fusion polypeptides (Table 1 and Fig. 3A, lanes 7 to 17). Mutants Δ51-56 and Δ103-108 bound to DNA like wild-type CBF-A (Fig. 3B, lanes 6 and 13). Several other mutants containing different internal deletions within the segment spanning residues 57 to 102 or within the segment spanning residues 109 to 142 did not bind DNA (Fig. 3B, lanes 7-12, 14, 15); one internal deletion mutant lacking residues 130 to 135 bound DNA very weakly (lane 16). These results, which are summarized in Table 1, demonstrate that the two CBF-A segments from 57 to 102 and from 109 to 142 are essential for DNA binding.

Domains of CBF-A that are necessary for interaction with CBF-C. Earlier evidence suggested that the interactions between the three subunits are important for DNA binding (40). In order to better understand these interactions, the CBF-A mutants were tested for their ability to form a heterodimeric CBF-A/CBF-C complex and a heterotrimeric CBF-B(CBF-A/CBF-C) complex. To test for CBF-A/CBF-C interactions, mutant GST-CBF-A polypeptides were incubated with radiolabeled wild-type CBF-C that was synthesized in a rabbit reticulocyte lysate, and the association between GST-CBF-A and CBF-C was detected by the GST pull-down assay (Fig. 4A). As expected, mutants 53-207, 53-142, Δ51-56, and Δ103-108, all of which bound DNA, interacted with CBF-C the way wild-type CBF-A did (Fig. 4A lanes 3 to 5, 8, and 15). One deletion mutant, Δ57-62, that did not bind DNA was, however,

able to interact with CBF-C (Fig. 4A, lane 9). In contrast, mutants 63-142, 53-120, and the internal deletion mutants within the segments spanning residues 63 to 99 and 112 to 135, which were defective in DNA binding, failed to interact with CBF-C in this assay (Fig. 4A, lanes 6, 7, 10 to 14, and 16 to 18). We conclude that the two segments of CBF-A between residues 63 and 102 and between residues 109 and 142 were essential for interactions with CBF-C (Table 1).

Domains of CBF-A that are necessary for interactions with CBF-B and for formation of a CBF-DNA complex. To determine whether a part of CBF-A in the CBF-A/CBF-C complex was essential for CBF-B association, the CBF-A mutants were also tested for their abilities to interact with CBF-B. The mutant GST-CBF-A polypeptides were first mixed with recombinant wild-type CBF-C and then incubated with in vitro-translated radiolabeled wild-type CBF-B. An association of CBF-B with GST-CBF-A in the presence of CBF-C was detected by the GST pull-down assay (Fig. 4B). Mutants 53-207, 53-142, Δ51-56, and Δ103-108, which formed a DNA-protein complex and interacted with CBF-C, also interacted with CBF-B as wild-type CBF-A did (Fig. 4B, lanes 3 to 5, 8, and 15). Deletion mutant Δ57-62, which did not bind to DNA but interacted with CBF-C, also interacted with CBF-B (Fig. 4B, lane 9). The other mutants containing different internal deletions in the

FIG. 3. Generation of amino acid deletion mutants of CBF-A and DNA binding of these mutants. (A) SDS-PAGE. The amino-terminal, carboxyl-terminal, and internal CBF-A deletions were expressed as GST-fusion proteins. Ten microliters of purified GST (lane 1), wild-type GST-CBF-A (lane 2), or GST-CBF-A mutants (lanes 3 to 17) was run on an SDS-12% polyacrylamide gel and stained with Coomassie blue. The locations of molecular mass markers, which were run alongside in the same gels, are indicated on the left. The amino acid residues of CBF-A that were deleted in the internal-deletion mutants are indicated with a Δ sign preceding their positions (lanes 7 to 17). For the amino-terminal and carboxyl-terminal deletions, the numbers indicate the amino acid residues that were included (lanes 3 to 6). (B) DNA binding of the CBF-A mutants. Twenty-five nanograms of wild-type (lane 1) and mutant CBF-A (lanes 2 to 16) were used in the gel shift assay. Each reaction mixture contained rCBF-C (20 ng) and rCBF-B (20 ng).

FIG. 4. Protein-protein interaction studies of the CBF-A mutants with CBF-C and CBF-B. (A) Interaction of the CBF-A mutants with CBF-C. In vitro-translated ^{35}S -labeled CBF-C (4 μl) was incubated in separate reaction mixtures with 500 ng of GST (lane 2), wild-type (wt) GST-CBF-A (lane 3), or GST-CBF-A mutants (lanes 4 to 18) as indicated. Lane 1 shows the input amount of in vitro-translated ^{35}S -labeled CBF-C that was used for each reaction. (B) Interaction of the CBF-A mutants with CBF-B in the presence of CBF-C. In vitro-translated ^{35}S -labeled CBF-B (2 μl) was used with the same CBF-A mutants used for the reaction shown in panel A. In each reaction GST-CBF-C (500 ng) was added to the reaction mixture. A smaller form of CBF-B appears in this interaction assay, probably because of proteolytic cleavage of CBF-B by proteases that copurify with bacterially made CBF-A and CBF-C.

segments between residues 63 and 99 and between residues 112 and 135 of CBF-A, which were defective in DNA binding and did not interact with CBF-C, failed to interact with CBF-B (Fig. 4B, lanes 6, 7, 10 to 14, and 16 to 18). Thus, the two segments of CBF-A between residues 63 and 102 and between residues 109 and 142, which were essential for DNA binding and for interaction with CBF-C, were also necessary for interactions with CBF-B. However, one cannot rule out the possibility that some of these mutations might result in an alteration of the three-dimensional structure of CBF-A, which in turn would account for the observed phenotype.

Previous evidence (40) as well as experiments presented here (Fig. 1) indicated that the formation of a CBF-A/CBF-C heterodimeric complex is needed for interactions with CBF-B. The finding that several internal deletions within CBF-A resulted in defective interactions with CBF-C suggested that these interactions utilized a large segment of CBF-A. However, this analysis did not allow us to determine whether specific residues were directly involved in interactions with CBF-B. To examine this, we generated a series of amino acid substitution mutations in CBF-A (Table 2). The amino acids that are evolutionarily conserved in the *S. cerevisiae* HAP3 polypeptide and rat CBF-A were selected for substitution mutations, and the substitutions were introduced in the full-length CBF-A. Mutant polypeptides were synthesized in *E. coli* as GST-fusion polypeptides. Among the 29 different amino acid substitution mutants that were generated, 16 were deficient in the formation of a DNA-protein complex whereas the other 13 were able to bind to DNA (Table 2 and Fig. 5).

Mutants defective in DNA binding were further tested for interactions with CBF-C and with CBF-B (Table 2 and Fig. 5). These mutants could be divided into three classes. The first group consisted of eight mutants that were unable to interact with either CBF-C or CBF-B, the second group consisted of two mutants that interacted with CBF-C but not with CBF-B, and the third group contained six mutants that interacted both with CBF-C and with CBF-B. Some of the class I mutants, T112A/I113R, F120R, and Y130G, failed to interact with CBF-C or CBF-B in the GST pull-down assays although they

TABLE 2. Properties of amino acid substitution mutants of CBF-A^a

CBF-A mutant ^b	DNA binding ^c	In vitro interaction ^d with:	
		CBF-C	CBF-B
Class I mutants			
A82K/P83G	—	—	—
V86R	—	—	—
T112A/I113R	±	—	—
F120R	±	—	—
Y130G	±	—	—
T112A/I113R/F120R	—	—	—
F120R/Y130G	—	—	—
T112A/I113R/Y130G	—	—	—
Class II mutants			
E92R	—	+	—
S99R	—	+	—
Class III mutants			
P60R	—	+	+
N63D	—	+	+
R66P	—	+	+
A79D	—	+	+
K80A	±	+	+
Q87E	—	+	+

^a Amino acid substitution mutants were expressed in *E. coli* as GST-fusion proteins, analyzed by SDS-PAGE to verify their predicted molecular masses, and tested for DNA binding in the presence of CBF-C and CBF-B as in Fig. 3. The protein-protein interaction assays of these mutants with CBF-C and CBF-B were examined as described in the legend to Fig. 4. The results of both tests are shown.

^b The nomenclature for each mutant designates the wild-type amino acid and its position, followed by the mutated amino acid that was introduced in place of the wild-type amino acid. In some cases more than one amino acid was substituted.

^c DNA binding is indicated as identical to wild-type CBF-A (+), no binding observed (—), or 5- to 10-fold reduced binding (±).

^d Interactions with CBF-C and CBF-B are indicated as wild-type interactions (+) or no interactions (—).

FIG. 5. Schematic representation of the mutations and their positions in context of the conserved domain of CBF-A containing residues 53 to 142. The following substitution mutations of CBF-A did not affect DNA binding: R53G, E54P, A71D/I72T, K77I, C85A, C89A, S102V/E108G, R110L, E116A, M122V, E132P, Y137D, and L138D/A144T. Symbols: ●, class I; △, class II; □, class III.

bind DNA weakly. This presumably represented the difference in the sensitivities of the two techniques used. We conclude that amino acid residues 82, 83, 86, 112, 113, 120, and 130, those altered in the first group of mutants, were most likely necessary for interactions with CBF-C, that residues 92 and 99, mutated in the second group of mutants, were essential for interaction with CBF-B, and that residues 60, 63, 66, 79, 80, and 87 were specifically required for interactions with DNA (Table 2 and Fig. 5).

Analysis of subunit interactions of CBF-A using the in vivo two-hybrid assay in *S. cerevisiae*. The subunit interactions of CBF-A were also examined in vivo by the yeast two-hybrid assay (12). Because homologs of the CBF subunits are present in yeast cells, we initially performed several control experiments to determine whether we could use this method as an in vivo protein-protein interaction assay to examine subunit interaction properties of CBF-A mutants. The conserved portion of CBF-A from residues 44 to 151 was cloned in frame downstream of the GAL4 DNA-binding domain (GAL4DB), whereas the segment of CBF-C from residues 27 to 123, which

together with CBF-A and CBF-B is needed for the formation of a CBF-DNA complex, was cloned in frame downstream of the GAL4 activation domain (GAL4AD). Both plasmids were transformed into a yeast strain bearing the *lacZ* gene under the control of a promoter with several GAL4 DNA-binding sites, and colonies were tested for the expression of *lacZ* with X-Gal.

Colonies containing either GAL4DB or GAL4AD were unable to activate *lacZ* transcription. Colonies containing either GAL4DB-CBF-A or GAL4AD-CBF-C alone were also negative by X-Gal staining. Similarly colonies containing a combination of either GAL4DB-CBF-A and GAL4AD or GAL4AD-CBF-C and GAL4DB did not stain with X-Gal. In contrast, yeast colonies containing the combination of GAL4DB-CBF-A and GAL4AD-CBF-C stained blue with X-Gal (data not shown). Hence, neither GAL4DB-CBF-A nor GAL4AD-CBF-C fusion polypeptides were able to activate *lacZ* transcription by themselves whereas the presence of the two fusion polypeptides together activated *lacZ* transcription. We interpret these results as indicating that CBF-A interacted with CBF-C in vivo in yeast cells, forming a GAL4DB-CBF-A/GAL4AD-CBF-C complex that activated *lacZ* transcription.

In our in vitro interaction assays, CBF-A did not interact with CBF-B in the absence of CBF-C. We also tested this interaction using the yeast two-hybrid assay. The conserved segment of CBF-B (residues 253 to 320) was cloned as a fusion polypeptide downstream of GAL4AD and expressed in yeast cells together with GAL4DB-CBF-A. Yeast colonies containing both plasmids were X-Gal positive, whereas colonies containing only the GAL4AD-CBF-B plasmid were X-Gal negative (data not shown). This indicates that CBF-A interacted with CBF-B in vivo in yeast cells. Our interpretation of this result is that in vivo HAP5 (33), the yeast homolog of CBF-C, mediated the interactions between CBF-A and CBF-B.

Several CBF-A mutants belonging to the three classes were then constructed in the GAL4DB plasmid and transformed in yeast together with the GAL4AD-CBF-C or with the GAL4AD-CBF-B plasmid (Table 3). Class I mutants $\Delta 71-77$, $\Delta 79-85$, $\Delta 87-93$, $\Delta 94-99$, $\Delta 112-119$, $\Delta 122-127$, V86R, and T112A/I113R/F120R did not develop blue yeast colonies in the presence of GAL4AD-CBF-C, indicating that these CBF-A mutants did not interact with CBF-C. Mutants $\Delta 63-70$ and $\Delta 130-135$ developed blue color only after long incubation, suggesting that these two mutants interacted weakly with CBF-C. Thus, mutants of CBF-A that were altered in their interactions with CBF-C in vitro were also defective in interactions with CBF-C in the two-hybrid assay in vivo. The mutants that were X-Gal negative in the presence of GAL4AD-CBF-C were also unable to form blue colonies in the presence of GAL4AD-CBF-B. This indicates that in vivo, the CBF-A mutants that were defective in interactions with CBF-C were also defective in interactions with CBF-B, presumably because of their inability to recruit HAP5.

In contrast, class II mutants E92R and S99R, which were X-Gal positive in the presence of GAL4AD-CBF-C, were X-Gal negative with GAL4AD-CBF-B, indicating that these CBF-A mutants were specifically defective in interacting with CBF-B. Importantly, our results show that CBF-A mutants that were defective in CBF-B interactions in vitro were also defective in vivo. Class III mutants $\Delta 57-62$, P60R, A79D, and Q87E, which developed blue colonies in the presence of GAL4AD-CBF-C, were also X-Gal positive in the presence of GAL4AD-CBF-B, indicating that these mutants interacted with both CBF-C and CBF-B in vivo. In summary, the parallel in vitro and in vivo interaction properties of three different

TABLE 3. In vivo interactions of CBF-A mutants with CBF-C and CBF-B in the yeast two-hybrid assay

GAL4DB-CBF-A mutant ^a	X-Gal staining result ^b	
	GAL4AD-CBF-C	GAL4AD-CBF-B
Class I mutants		
$\Delta 63-70$	±	ND
$\Delta 71-77$	—	—
$\Delta 79-85$	—	ND
$\Delta 87-93$	—	—
$\Delta 94-99$	—	—
$\Delta 112-119$	—	—
$\Delta 122-127$	—	—
$\Delta 130-135$	±	ND
V86R	—	—
T112A/I113R/F120R	—	—
Class II mutants		
E92R	+	—
S99R	+	—
Class III mutants		
$\Delta 57-62$	+	+
P60R	+	+
A79D	+	+
Q87E	+	+

^a Some of the CBF-A mutants that were defective in DNA binding (Tables 1 and 2) were used in the yeast two-hybrid system to study their interactions with CBF-C and CBF-B.

^b +, blue color of same intensity as wild-type CBF-A observed after 2 h; ±, blue color observed after 24 to 48 h; —, white color after 48 h; ND, not determined.

classes of CBF-A mutants suggest that CBF-A contains three functional interaction domains essential for DNA binding.

Mutants of CBF-A that form a CBF-A/CBF-C complex but do not bind DNA inhibit formation of the wild-type CBF-DNA complex. The proposed existence of three domains in CBF-A predicts that class II mutants which were able to form a CBF-A/CBF-C complex but were unable to interact with CBF-B and class III mutants which formed a heterotrimeric complex but were unable to bind DNA should inhibit DNA binding of wild-type CBF by forming an inactive CBF-A/CBF-C complex. In contrast, class I mutants which are defective in the formation of a CBF-A/CBF-C complex should not inhibit the DNA binding of wild-type CBF. To test this hypothesis, excess amounts of mutant CBF-A polypeptides were added to the DNA-binding reaction mixtures containing the three wild-type CBF subunits (Fig. 6A). Class III mutants P60R and A79D, which showed normal interactions both with CBF-C and with CBF-B but did not bind DNA, inhibited the DNA binding of wild-type CBF subunits (Fig. 6A, lanes 2 to 5). Class II mutants E92R and S99R, which interacted with CBF-C but failed to interact with CBF-B and did not bind DNA, also inhibited DNA binding (Fig. 6A, lanes 8 to 11). Hence, these mutants acted as dominant negative mutants. In contrast, class I mutants V86R and F120R/Y130G, which were defective in interactions with CBF-C, did not inhibit DNA binding (Fig. 6A, lanes 6, 7, 12, and 13). Interestingly, the inhibition of DNA binding by the mutant polypeptides was suppressed by the addition of excess CBF-C to the reaction mixtures (Fig. 6B, lanes 4 and 8) but not by the addition of CBF-B (lanes 3 and 7) or of CBF-A (data not shown), indicating that the mutants inhibited DNA binding by sequestering CBF-C. Whereas the inhibition by mutant E92R was fully suppressed by the addition of excess CBF-C (Fig. 6B, lane 4), inhibition by mutant P60R was partially suppressed by the addition of excess CBF-C and was fully

FIG. 6. Inhibition of CBF-DNA complex formation by some CBF-A mutants. (A) Gel shift assays were performed with some of the mutant GST-CBF-A (Table 2). Reaction mixtures in all lanes contain wild-type GST-CBF-A (10 ng) and 20 ng each of GST-CBF-C and GST-CBF-B. GST-CBF-A mutants in excesses of $10\times$ (100 ng) and $20\times$ (200 ng) were added in lanes 2 to 13 as indicated. For each reaction (lanes 2 to 13), CBF-A mutants were incubated with GST-CBF-B, GST-CBF-C, and labeled oligonucleotide for 10 min at room temperature. The reaction was started with the addition of wild-type GST-CBF-A, further incubated for 10 min, and then run on a 5% polyacrylamide gel. The gel was subsequently dried and autoradiographed. (B) Suppression of the inhibition by CBF-A mutants by CBF-C, CBF-B, or both. Gel shift assays with the two mutants was done as in panel A. Excess GST-CBF-C (200 ng) and GST-CBF-B (200 ng) were added to the reaction mixture after incubation with GST-CBF-A. After an additional 10-min incubation, the reaction mixture was run on a 5% polyacrylamide gel. The reaction mixture in all lanes contained 10 ng of wild-type GST-CBF-A and 20 ng each of GST-CBF-C and GST-CBF-B.

suppressed by the addition of both CBF-C and CBF-B (lanes 8 and 9). We interpret this result as indicating that the class II mutant sequestered only CBF-C, whereas the class III mutant sequestered both CBF-C and CBF-B. Inhibition by the class III

mutant was not suppressed by CBF-B alone (Fig. 6B, lane 7), indicating that the mutant CBF-A did not sequester or interact with CBF-B without also sequestering CBF-C.

The evolutionarily conserved segment of CBF-A shows similarities with the conserved segment of CBF-C, and both CBF segments show similarities with an ancient histone-like motif. The segment of CBF-A that is necessary for subunit interactions and DNA binding has been highly conserved during evolution (Fig. 7A). This segment did not show any sequence similarity with any other known motifs that have been described for other heteromeric transcription factors. Recently, a yeast homolog of CBF-C, designated HAP5, was also isolated, and the segment of CBF-C needed for DNA binding showed a high degree of sequence identity with part of HAP5 (Fig. 7B). Surprisingly, these conserved segments in CBF-A and CBF-C showed similarities with the globular domains of histones H2B and H2A, respectively (Fig. 7A and B). Interestingly, the conserved segments of CBF-A and CBF-C showed a high degree of sequence homology (Fig. 7C). Furthermore, these CBF segments were also similar to the archaeobacterial histone-like protein Hmf-2. This suggests that these two CBF subunits originated from a common ancestor, perhaps a precursor of the archaeobacterial Hmf-2 polypeptide.

Hmf-2 forms a nucleosome-like DNA-protein complex, and the amino acid sequence of Hmf-2 is similar to that of various histones (39). Hence, we designated the region of sequence similarity among Hmf-2, CBF-A, and CBF-C a histone-like motif. Importantly, the C-terminal portion of this motif is present in Dr1, a human phosphoprotein associated with the TATA-binding protein TBP (Fig. 7A). Dr1 itself does not bind to DNA, but after it associates with TBP, it represses both basal and activated transcription of class II genes (20).

Recent structural studies of the histone octamer indicated that the segments of the amino acid residues of both histones H2A and H2B, which are shown in Fig. 7, have a characteristic secondary structure, called the histone fold, that represents the central portion of each histone within the H2A/H2B dimer (1, 2). A similar histone-fold structure is also found in the other two histones, H3 and H4, within the H3/H4 dimer (half-tetramer), in spite of limited sequence homologies between these histones. X-ray crystallographic analysis has established that within the histone dimers, the folded histone chains interact by clasping each other in what has been described as a molecular handshake motif (1). Unlike dimers between two globular domains that have small local contacts, the histone dimers have extensive molecular contacts. The histone fold, which is the common feature of the four histones, consists of a short α -helix followed by a loop and a β -strand segment, a long helix, another short loop and β -strand segment, and a short helix. It has been hypothesized that the histone fold arose as the result of a tandem, parallel duplication of the helix-strand-helix motif (2, 34). The sequence homologies between CBF-A and H2B and between CBF-C and H2A suggest that the nature of the CBF-A/CBF-C interactions might be similar to those of the H2A/H2B dimer.

DISCUSSION

To better understand the potential interactions among the three subunits of CBF and between these subunits and DNA, we first determined the stoichiometries of the CBF subunits in CBF and in the CBF-DNA complex. Our cross-linking experiments established that one molecule of each subunit is present in CBF. DNA-binding experiments with full-length and truncated subunits were similarly consistent with the presence of one molecule of each subunit in the CBF-DNA complex.

FIG. 7. Alignment of the amino acid sequences of rat CBF-A (accession no. J05701) and CBF-C (accession no. U17607) with yeast homologs and other sequences. The evolutionarily conserved segment of CBF-A and CBF-C was compared with sequences from the SwissProt database by using the Blastp program from the Genetics Computer Group (University of Wisconsin, Madison). Identical amino acids are shown between the sequences, and conservative amino acid changes are indicated by a plus sign. The numbers to the left and right of the sequences indicate the first and last residues of the segment of each polypeptide that is used in the comparative analysis. (A) Homologs of CBF-A. *S. cerevisiae* HAP3 (17) shows 83% amino acid sequence similarity to CBF-A in the region shown. This portion of the CBF-A sequence is identical in rats, mice, and humans (26). Histone H2B from various eukaryotic species (45) showed 50% amino acid sequence similarity to CBF-A. In this figure the amino acid sequence of part of mouse histone H2B is shown. A portion of Dr1, the human TBP-associated protein (20), shows 71% amino acid sequence similarity to CBF-A, whereas HMF-2, the DNA-binding protein from the hyperthermophilic archaeon *Methanothermobacter fervidus* (39), shows 54% amino acid sequence similarity. (B) Homologs of CBF-C. Between residues 42 and 115, CBF-C shows 80% amino acid sequence similarity to *S. cerevisiae* HAP5 (33) and 44% and 48% amino acid sequence similarity to histone H2A from various eukaryotic species (45) and to HMF-2, respectively. In this figure the amino acid segment of part of mouse histone H2A is shown. (C) Alignment of the amino acid sequences of CBF-C, CBF-A, and HMF-2. CBF-C shows 47% amino acid sequence similarity to CBF-A in the segments of the polypeptides that are shown. At the time this communication was submitted, Baxevanis et al. (3) reported similar findings regarding sequence similarities between histone-fold motifs and other DNA-binding proteins, including CBF-A and CBF-C.

The sequence of CBF-A between residues 53 and 142 is strongly conserved in *S. cerevisiae* and mammals. Our deletion analysis of CBF-A first demonstrated that two segments between residues 57 and 102 and between residues 109 and 142 were needed for the formation of a heterotrimeric CBF-DNA complex. Amino acid substitution mutations in the conserved segment revealed the existence of three classes of mutants, the properties of which were identical in both *in vitro* and *in vivo* experiments. These three classes of mutants demonstrated the existence of three steps in the formation of the CBF-DNA complex and defined three possible domains of CBF-A (Fig. 8). One class of mutants was defective (i) in interactions with

FIG. 8. Proposed three interaction domains and histone-fold motif of CBF-A. The three domains of CBF-A are shown as defined by the three classes of mutants and are based on both amino acid substitution and deletion mutations (see the text). The histone-fold motif is defined by the amino acid sequence homology with histone H2B (Fig. 7). The different domains are all present within the conserved segment of CBF-A from residues 53 to 142. The positions of amino acid residues covering each domain are indicated within the parentheses.

CBF-C, (ii) in the formation of the CBF-B(CBF-A/CBF-C) heterotrimer, and (iii) in the formation of the CBF-DNA complex. In the second class of mutants, the CBF-A/CBF-C interactions were normal, but the interactions between the dimeric CBF-A/CBF-C complex and CBF-B were defective and the CBF-DNA complex failed to form. In the third class of mutants, both the formation of the heterodimeric CBF-A/CBF-C complex and of the heterotrimeric CBF-B(CBF-A/CBF-C) complex occurred normally, but binding to DNA was defective. Hence, the formation of a CBF-A/CBF-C dimeric complex is an essential first step in the assembly of the CBF-DNA complex. After a CBF-A/CBF-C heterodimer is formed, a heterotrimer with CBF-B is formed and only then can CBF bind to DNA. Our cross-linking results were also entirely consistent with the existence of three successive steps in the assembly of the CBF-DNA complex.

A major portion of the evolutionarily conserved segment of CBF-A between amino acid residues 63 to 102 and between residues 109 to 142, was essential for interaction with CBF-C, suggesting that CBF-A makes extensive contacts with CBF-C (Fig. 8). Interestingly, the amino acid residues between 64 to 125 of CBF-A showed sequence similarities with a portion of histone H2B which consists of a characteristic histone-fold secondary structure. Thus the CBF-C interaction domain and the histone-fold motif of CBF-A largely overlap each other. Although the nature of the interaction between CBF-A and CBF-C will be better understood after the completion of similar studies with CBF-C, which also showed sequence similarities with the histone H2A, our study suggests that the interface within the CBF-A/CBF-C heterodimer is similar to the molecular handshake motif characteristic of the H2A/H2B interface. Our previous studies had shown that the mammalian CBF-A

and CBF-C copurify after several chromatographic steps, indicating that native CBF-A and CBF-C form a stable complex in vivo. This complex holds together tightly, since SDS or high concentrations of guanidine-hydrochloride are required to dissociate the native subunits (30).

The site of interactions with CBF-B was identified by two point mutations at residues 92 and 99 in CBF-A. The segment covered by these mutations physically overlaps with the CBF-C interaction domain. Since CBF-B did not interact separately with CBF-A but rather interacted exclusively with the CBF-A/CBF-C complex, the interactions of CBF-A with CBF-C must generate an interface that interacts with CBF-B. Thus the CBF-B interaction domain in CBF-A could be part of a common interface in the CBF-A/CBF-C complex to which CBF-C also contributes. This view is supported by the complete absence of CBF-A/CBF-B and CBF-C/CBF-B dimeric species in our cross-linking experiments. In these reactions only the CBF-A/CBF-C heterodimer and the CBF-B(CBF-A/CBF-C) heterotrimer were observed. It is nonetheless possible that this domain of CBF-A for CBF-B interactions is involved in changing the conformation of CBF-C, which then would interact with CBF-B. In addition, we cannot exclude the possibility that other residues besides 92 and 99 are also involved in the interaction with CBF-B.

The identification of a domain in CBF-A that is specifically needed for interactions with DNA was based on the properties of the third class of CBF-A mutants. This domain overlaps with a large portion of the CBF-C interaction domain. Potential functional domains of HAP3, the yeast homolog of CBF-A, were recently examined by in vivo genetic analysis of *S. cerevisiae* (47). Although this analysis did not take into account the role of HAP5, the yeast homolog of CBF-C, in the formation of the HAP/DNA complex, this study showed the existence of a DNA-binding domain in HAP3 that corresponds to part of the DNA binding domain we identified in CBF-A.

In our study, CBF-A mutants containing mutations in the CBF-B interaction domain and in the DNA-binding domain inhibited the DNA binding of wild-type CBF subunits. These CBF-A mutants competed with wild-type CBF-A to interact with CBF-C, and interactions of CBF-A mutants with CBF-C resulted in the formation of a CBF-A/CBF-C heterodimer that was inactive in DNA binding. This indicates that mutations in these domains of CBF-A did not interfere with its interactions with CBF-C. Because these CBF-A mutants act as dominant negative mutants, they could be used to inactivate CBF in mammalian cells and will be valuable tools for exploring the cellular role of CBF in transcription activation.

In many heteromeric transcription factors, the segment needed for DNA binding consists of two modular domains, one for subunit interactions and one for DNA binding. A similar modular arrangement is found in the segment of CBF-B needed for DNA binding, but in CBF-A the three functional domains are not separated from each other but rather overlap. This property distinguishes CBF-A from CBF-B and from many other heteromeric transcription factors. Interestingly, the sequence homologies between the segment of CBF-A that is needed for DNA binding and histone H2B strongly suggest that the secondary structure of this segment of CBF-A is similar to the characteristic histone fold. That the segment of CBF-C needed for DNA binding has similarities with the equivalent segment of CBF-A and with histone H2A suggests that this CBF subunit also contains a histone-fold secondary structure. X-ray crystallographic analysis of the histone octamer has indicated that the histone-fold motifs are utilized in the formation of histone dimers and also provide surfaces to contact DNA in nucleosome-DNA complexes (1). The homol-

ogies between histones and Hmf-2, which forms nucleosome-like structures in archaeobacteria, suggest that the histone-fold structure has been functionally conserved during evolution. The homologies between the CBF subunits and histones imply that the mechanism by which these CBF subunits interact with each other to form dimers is similar to the mechanism by which histones H2A and H2B interact with each other in H2A/H2B dimers. We further speculate that the mechanisms whereby the CBF-A/CBF-C dimer contacts DNA in the CBF-DNA complex might be similar to those utilized by the H2A/H2B dimer in its interactions with DNA within the nucleosomal octamers. Whereas the CBF-DNA complex is a sequence-specific DNA-protein complex, nucleosome interaction with DNA is not sequence specific. This allows for the possibility that in the CBF-DNA complex, the CBF-A/CBF-C dimer makes nonspecific contacts with DNA, whereas CBF-B makes sequence-specific DNA contacts. Evidence that CBF-B makes sequence-specific DNA contacts comes from an allele-specific mutation in the DNA-binding domain of HAP2, the yeast homolog of CBF-B. This mutant is defective in the formation of a HAP-DNA complex with wild-type DNA, instead favoring the formation of a HAP-DNA complex with a mutant DNA containing a CCAAC motif instead of CCAAT (47). A similar observation was also made when an identical mutation was introduced in CBF-B (8a).

It is also possible that the histone-fold motifs of these subunits may also have a role in transcription activation by CBF. CBF binds to the promoters of many cellular genes, and the CBF-binding site is critical for the in vitro and in vivo transcriptional activity of these promoters (9, 21, 29, 32, 46). Two recently isolated *Drosophila* TBP-associated factors (TAFs), p62 and p42, also showed amino acid sequence similarities with the histone-fold motifs of histones H4 and H3, respectively (23). TAFs are essential cofactors for the regulated transcription of class II promoters (42). On the basis of the model whereby histone dimers interact with each other in the histone octamer, we speculate that the CBF-A/CBF-C dimer could interact with some TAFs or other TBP-associated proteins such as Dr1 to activate or derepress transcription. One other possibility is that the CBF-A/CBF-C dimer could also disrupt the chromatin structure of promoters by interfering with the association of the histone H2A/H2B dimer in nucleosomes. Additional studies are needed to test these hypotheses.

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