Determination of Functional Domains in the C Subunit of the CCAAT-Binding Factor (CBF) Necessary for Formation of a CBF-DNA Complex: CBF-B Interacts Simultaneously with both the CBF-A and CBF-C Subunits To Form a Heterotrimeric CBF Molecule

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The mammalian CCAAT-binding factor (CBF; also called NF-Y and CP1) is a heterotrimeric protein consisting of three subunits, CBF-A, CBF-B, and CBF-C, all of which are required for DNA binding and all of which are present in the CBF-DNA complex. In this study using cross-linking and immunoprecipitation methods, we first established that CBF-B interacts simultaneously with both subunits of the CBF-A–CBF-C heterodimer to form a heterotrimeric CBF molecule. We then performed a mutational analysis of CBF-C to define functional interactions with the other two CBF subunits and with DNA using several in vitro assays and an in vivo yeast two-hybrid system. Our experiments established that the evolutionarily conserved segment of CBF-C, which shows similarities with the histone-fold motif of histone H2A, was necessary for formation of the CBF-DNA complex. The domain of CBF-C which interacts with CBF-A included a large portion of this segment, one that corresponds to the segment of the histone-fold motif in H2A used for interaction with H2B. Two classes of interactions involved in formation of the CBF-A–CBF-C heterodimer were detected; one class, provided by residues in the middle of the interaction domain, was needed for formation of the CBF-A–CBF-C heterodimer. The other, provided by sequences flanking those of the first class was needed for stabilization of the heterodimer. Two separate domains were identified in the conserved segment of CBF-C for interaction with CBF-B; these were located on each side of the CBF-A interaction domain. Since our previous experiments identified a single CBF-B interaction domain in the histone-fold motif of CBF-A, we propose that a tridentate interaction domain in the CBF-A–CBF-C heterodimer interacts with the 21-amino-acid-long subunit interaction domain of CBF-B. Together with our previous mutational analysis of CBF-A (S. Sinha, I.-S. Kim, K.-Y. Sohn, B. de Crombrugge, and S. N. Maity, Mol. Cell. Biol. 16:328–337, 1996), this study demonstrates that the histone-fold motifs of CBF-A and CBF-C interact with each other to form the CBF-A–CBF-C heterodimer and generate a hybrid surface which then interacts with CBF-B to form the heterotrimeric CBF molecule.

The mammalian CCAAT-binding factor (CBF; also called NF-Y and CP1) is a DNA-binding protein that binds to CCAAT motifs in the promoters of numerous eukaryotic genes (6, 7, 9, 13, 17, 20). In several promoters, mutations of the CCAAT motif which abolish CBF binding decreased the level of transcriptional activity (8, 14, 22, 25, 31), indicating that CBF is a transcriptional activator.

CBF consists of three different subunits, CBF-A, CBF-B, and CBF-C (18). Using recombinant polypeptides, we demonstrated that all three subunits are needed for DNA binding and that one copy of each is present in the CBF molecule and in the CBF-DNA complex (26, 27). The A and B subunits of CBF in rats, mice, and humans show almost 99% amino acid sequence identity, on the basis of cDNA-derived sequences (5, 15, 19, 28, 29). Furthermore, a segment in CBF-A, one in CBF-B, and one in CBF-C show a high degree of sequence identity with segments of the Saccharomyces cerevisiae HAP3, HAP2, and HAP5 polypeptides, respectively. These three polypeptides are subunits of a multimeric yeast CCAAT-binding factor that regulates expression of several nuclear genes essential for mitochondrial function (11, 21, 24).

Using an in vitro protein-protein interaction assay, we showed that CBF-A and CBF-C first interact with each other to form a CBF-A–CBF-C heterodimer, which then interacts with CBF-B to form the heterotrimeric CBF molecule (26, 27). Mutational analysis of CBF-B demonstrated that the conserved segment of this subunit necessary for DNA binding consists of two modular domains, a domain for interactions with the CBF-A–CBF-C complex and a DNA-binding domain (16). Similar conclusions were also reached by mutational analysis of the yeast HAP2 polypeptide (23, 32). Recently, a mutational analysis of CBF-A showed that the conserved segment of the subunit necessary for DNA binding consisted of three functional domains, a domain for interaction with CBF-C, a domain for interaction with CBF-B, and a DNA-binding domain (26). Unlike the modular arrangement of the two interaction domains in CBF-B, the three domains in CBF-A overlapped each other. Interestingly, the major portion of the conserved CBF-A segment showed sequence similarity with the histone-fold motif of H2A. This suggested that a histone-fold-like structure in CBF-A mediated the interactions with other CBF subunits and with DNA. The conserved segment of CBF-C also showed amino acid sequence similarity with the histone-fold motif of H2A. However, the functional domains by which CBF-C participates in formation of the heterotri-
meric CBF molecule and the CBF-DNA complex remain to be established.

In this study, we analyzed the interactions among the three CBF subunits by cross-linking and coimmunoprecipitation methods. This study revealed that CBF-B makes contact simultaneously with both CBF-A and CBF-C in the CBF-A–CBF-C heterodimer, to form the heterotrimeric CBF molecule. To determine the role of CBF-C in formation of the CBF-DNA complex, we performed a mutational analysis of this subunit. This analysis defined domains within the conserved histone-fold motif of CBF-C that are necessary for DNA binding and for interactions with CBF-A and with CBF-B. Together with our previous analysis, this study establishes a model by which the three CBF subunits form the heterotrimeric CBF and the CBF-DNA complex.

MATERIALS AND METHODS

Generation of mutants of CBF-C. The amino- and carboxyl-terminal deletions of CBF-C polypeptides were generated by a PCR approach. An EcoRI site for the N-terminal deletions and a XhoI site for the C-terminal deletions were created at the 5′-ends of oligonucleotides used to generate specific deletion. DNA stretches corresponding to residues 44 to 151 of CBF-A and contains a tryptophan marker, and pACT-II, which contains a leucine marker.

Results

Interaction between CBF-B and the CBF-A–CBF-C heterodimer examined by cross-linking and coimmunoprecipitation. Previously, we established that CBF-A and CBF-C together form a CBF-A–CBF-C heterodimer and that CBF-B does not interact with either CBF-A or CBF-C separately but rather associates with the CBF-A–CBF-C heterodimer to form the heterotrimeric CBF. However, it is not known whether CBF-B associates with both subunits of the CBF-A–CBF-C heterodimer or with only one of these subunits. To analyze near-neighbor contacts of the three subunits of CBF, we analyzed the subunit interactions by chemical cross-linking using different cross-linkers, glutaraldehyde and DMS. The three CBF subunits were first synthesized in vitro in a rabbit reticulocyte lysate. CBF-B was labeled during synthesis, whereas CBF-A and CBF-C, which were synthesized together in a single reaction, were unlabeled. CBF-A and CBF-C together formed a stable heterodimer after having been synthesized together in the reticulocyte lysate.

The labeled CBF-B was incubated with the CBF-A–CBF-C heterodimer, and the mixture was cross-linked with different concentrations of glutaraldehyde (Fig. 1A, lanes 3 to 6) or with different concentrations of DMS (lanes 9 to 11). To monitor specific interactions of CBF-B with the CBF-A–CBF-C heterodimer, the cross-linked mixture was immunoprecipitated with anti-CBF-A antibody. No labeled CBF-B was coimmunoprecipitated with the anti-CBF-A antibody after CBF-B was cross-linked with glutaraldehyde in the absence of CBF-A and CBF-C (Fig. 1A, lane 2). CBF-B was also not immunoprecipitated with the anti-CBF-A antibody after it was cross-linked in the presence of either CBF-A alone or CBF-C alone (data not shown). In contrast, the labeled CBF-B was coimmunoprecipitated with the antibody after cross-linking with glutaraldehyde in the presence of both recombinant CBF-A and CBF-C (Fig. 1A, lanes 3 to 6), indicating that CBF-B formed a complex with the CBF-A–CBF-C heterodimer. In these reactions, in addition to labeled CBF-B, three different labeled molecular mass species (designated here X, Y, and Z) were also coimmunoprecipitated. Similarly, these three discrete species were also coimmunoprecipitated after CBF-B was cross-linked with DMS in the presence of both CBF-A and CBF-C (Fig. 1A, lanes 9 to 11). The three cross-linked species were also observed when the coimmunoprecipitations were performed with anti-CBF-C antibody instead of the anti-CBF-A antibody (data not shown).

We conclude from these results that the three species are cross-linked products and were generated as a result of interaction of CBF-B with the CBF-A–CBF-C heterodimer. Whereas the amounts of the three cross-linked species formed with DMS were unchanged with increasing concentrations of DMS (Fig. 1A, lanes 9 to 11), the relative amounts of each of the three species formed with glutaraldehyde varied with increasing concentrations of glutaraldehyde (lanes 3 to 6). The amounts of X and Y decreased, but the amount of Z increased with increasing concentrations of glutaraldehyde. DMS, which consists of an 11-Å (1.1-nm) spacer arm, cross-links two strongly interacting polypeptides. In contrast, glutaraldehyde, which forms oligomers or polymers in aqueous solution, cross-links both strongly and weakly interacting polypeptides and also can cross-link multiple polypeptides in a higher-order complex (30). We interpret the cross-linking result as indicat-
ing that Z is a higher-order complex and that X and Y are subsets of the Z species.

Previously we determined that a truncated form of CBF-B containing its conserved subunit interaction domain interacted with the CBF-A–CBF-C complex. If the three species X, Y, and Z were the results of interactions between the subunit interaction domain of CBF-B and the CBF-A–CBF-C heterodimer, then these three species should have lower molecular masses than those formed with the wild-type CBF-B when a truncated form of CBF-B containing an intact subunit interaction domain is used. A deleted form of CBF-B (B210-330) containing the conserved segment was synthesized in the rabbit reticulocyte lysate and labeled during synthesis. The labeled B210-330 was coimmunoprecipitated with the anti-CBF-A antibody after cross-linking with glutaraldehyde in the presence of both CBF-A and CBF-C (Fig. 1B, lane 1). Three cross-linked species were coimmunoprecipitated together with the un-cross-linked B210-330, but the molecular mass of each of the three cross-linked species was correspondingly lower than those formed with full-length CBF-B (Fig. 1B, lane 2). The decreases were consistent with the decrease in the molecular mass of CBF-B resulting from the deletion. This result suggested that the three cross-linked species were generated by specific interactions of the subunit interaction domain of CBF-B with the CBF-A–CBF-C heterodimer.

Labeled CBF-B could be cross-linked either with CBF-A, with CBF-C, or with both subunits to form the three cross-linking species. To test this possibility, a deleted form of CBF-A (A1-160) and two deleted forms of CBF-C (C1-175 and C27-119) were generated. The deletions were performed in the region of the subunits which are not essential for DNA binding. The deleted CBF-A was synthesized in the rabbit reticulocyte lysate together with full-length CBF-C, and each of the deleted CBF-Cs was synthesized together with full-length CBF-A. Each of the cosynthesized subunits together formed a heterodimeric complex (data not shown), which interacted with CBF-B as assayed by DMS cross-linking followed by coimmunoprecipitation with the anti-CBF-A antibody. In each reaction, cross-linking with CBF-B, as with full-length CBF-A and CBF-C, resulted in the formation of three different molecular weight species (Fig. 1B, lanes 3 to 5; in lanes 4 and 5, a cross-linked species of higher molecular mass was observed after longer exposure of the autoradiogram). Among the three cross-linked species formed with CBF-A–C1-175 (Fig. 1B, lane 3), both Y and Z were replaced with species of lower molecular masses than those formed with full-length CBF-C (lane 2) whereas the X species remained unchanged. Similarly, one of

![FIG. 1. Analysis of interaction between CBF-B and the CBF-A–CBF-C heterodimer by cross-linking and coimmunoprecipitation methods. (A) Cross-linking of the CBF subunits with glutaraldehyde and DMS. The CBF subunits were expressed by in vitro transcription-translation. CBF-B was labeled, and CBF-A and CBF-C were cotranslated and were not labeled. One microliter of the labeled CBF-B (B* in panel B) and 1 μl of the cotranslated CBF-A and CBF-C (+A/C) were first cross-linked either by different concentrations of glutaraldehyde (0.03% [lane 3], 0.01% [lane 4], 0.005% [lane 5], 0.0025% [lane 6], and 0% [lane 7]) or by different concentrations of DMS (4 mg/ml [lane 9], 2 mg/ml [lane 10], 1 mg/ml [lane 11], and 0 mg/ml [lane 12]), and the cross-linked mixtures were then immunoprecipitated with the anti-CBF-A antibody. The immunoprecipitated materials were run on a 4 to 15% gradient gel. For the control experiment, the labeled CBF-B was cross-linked in the absence of CBF-A and CBF-C with 0.02% glutaraldehyde (lane 2) or with 4 mg of DMS per ml (lane 8) and then immunoprecipitated. Lane 1 shows the input of the labeled CBF-B in each reaction. Molecular mass markers were run alongside in the same gels, and their sizes are noted at the left. The bands X, Y, and Z represent the cross-linked products. The labeled bands in lanes 1 to 7 and in lanes 8 to 12 were detected after 16 and 36 h of exposure, respectively. (B) Cross-linked complexes formed with different-sized variants of CBF subunits or with mutants of CBF-A. A truncated form of CBF-B, B210-330 (B210-330*), was expressed and labeled by in vitro transcription-translation. The labeled B210-330 was cross-linked with DMS (2 mg/ml) in the presence of the cotranslated A/C and immunoprecipitated with the anti-CBF-A antibody (lane 1). Each of two truncated forms of CBF-C, C1-175 and C27-119, was cotranslated with full-length CBF-A, and a truncated form of CBF-A, A1-160, was cotranslated with full-length CBF-C. Full-length and size variants of the cotranslated CBF-A–CBF-C as indicated in lanes 2 to 5 were cross-linked with the labeled full-length CBF-B with DMS (2 μg/ml) and then immunoprecipitated. Two CBF-A mutants, E92R (lane 6) and S99R (lane 7), which were cotranslated with full-length CBF-C, were cross-linked with the labeled full-length CBF-B with 0.015% glutaraldehyde and then immunoprecipitated. (C) Lanes 2 to 5 of panel B are schematically represented here in lanes 1 to 4, respectively. (D) CBF subunit composition of the three different cross-linked complexes, X, Y, and Z, that were formed with CBF-B.
the three cross-linked species formed with CBF-A–C27-119 had the same molecular mass as the X species, while the other two had lower molecular masses (lane 4). The only difference between the results of the reactions shown in lane 2 and those shown in lanes 3 and 4 is the molecular mass of CBF-C, which was decreased as a result of the deletions. Our interpretation of this result is that both Y and Z contain CBF-C but X does not. In contrast, one of the cross-linked species formed with A1-160–CBF-C (lane 5) had the same molecular mass as Y of lane 2, while the other two had lower molecular masses than X and Z, suggesting that X and Z contain CBF-A but Y does not. The cross-linked species in lanes 2 to 5 of Fig. 1B are schematically represented in Fig. 1C. The results suggest that X is a cross-linked complex of CBF-B with CBF-A, that Y is a complex of CBF-B with CBF-C, and that Z is a complex of CBF-B with both CBF-A and CBF-C (Fig. 1D). We conclude that CBF-B makes contact with both the CBF-A and CBF-C subunits of the CBF-A–CBF-C heterodimer.

Recently, we characterized two amino acid substitution mutants of CBF-A (A92 and A99), which interacted with CBF-C to form a CBF-A–CBF-C heterodimer but were unable to interact with CBF-B (26). Each CBF-A mutant was synthesized in the reticulocyte lysate together with the wild-type CBF-C, cross-linked in the presence of labeled CBF-B, and communoprecipitated with the anti-CBF-A antibody (Fig. 1B, lanes 6 and 7). In this experiment, no un-cross-linked CBF-B or any of the three cross-linked species were observed in the immunoprecipitate. Similarly, mutational analysis of CBF-C, which is described later in this paper, identified several mutants of CBF-C which interacted with CBF-A to form a heterodimer but did not interact with CBF-B; these mutants did not form any of the three cross-linked species. Thus, the properties of these CBF-A and CBF-C mutants, which inhibit the binary interactions between CBF-B and the CBF-A–CBF-C heterodimer, indicate that CBF-B cannot interact with CBF-A if it cannot interact at the same time with CBF-C, and similarly it cannot interact with CBF-C without interacting with CBF-A at the same time.

**Segment of CBF-C necessary for DNA binding.** To determine which segment of CBF-C was necessary for DNA binding, we generated a series of amino- and carboxyl-terminal deletion mutants in the cDNA of the CBF-C subunit (Fig. 2). The mutants were expressed in *E. coli* as fusion polypeptides with GST. The purified polypeptides (Fig. 3A) were tested in DNA-binding reactions in the presence of recombinant CBF-A and recombinant CBF-B. Like wild-type CBF-C, the deletion mutants 1-175, 1-119, and 34-175 bound to DNA (Fig. 3B, lanes 2, 3, and 6). A deletion mutant of CBF-C containing amino acid residues 34 to 119 also bound to DNA like wild-type CBF-C (data not shown). In contrast, mutants 1-110 and 40-119 did not bind DNA (Fig. 3B, lanes 4 and 5). This result indicates that amino acids between residues 34 and 119 of CBF-C are necessary for DNA binding. This segment of CBF-C showed a high degree of amino acid sequence identity with a segment of the yeast HAP5 polypeptide (21).

To analyze the segment of CBF-C in detail, a series of in-frame internal deletion mutants were generated as GST fusion polypeptides (Fig. 2). Mutant Δ116-120 bound to DNA like wild-type CBF-C (Fig. 3B, lane 16). Several other mutants containing internal deletions within the segment spanning residues 47 to 74 or within the segment spanning residues 92 to 113 did not bind DNA (Fig. 3B, lanes 8 to 11 and 13 to 15). Two mutants, Δ42-45 and Δ85-90, bound DNA very weakly (Fig. 3B, lanes 7 and 12). A series of amino acid substitution mutations within the conserved segment were also generated as GST fusion polypeptides. The amino acids which were conserved between CBF-C and HAP5 were mutated. Nine mutants containing amino acid substitutions within the segment spanning residues 46 to 115 did not bind to DNA (see Table 3). These experiments confirm that specific amino acids within the segment of CBF-C from residues 34 to 119 are necessary for DNA binding.

Our recent studies have demonstrated that formation of the CBF-DNA complex occurred in three sequential steps: (i) interactions between CBF-A and CBF-C to form a CBF-A–CBF-C heterodimer, (ii) interactions between CBF-B and the CBF-A–CBF-C heterodimer to form the heterotrimer CBF, and (iii) binding of CBF to DNA (26). To determine the domains of CBF-C involved in the first two steps, mutants of CBF-C were tested in subunit interactions with both CBF-A and CBF-B.

**Domains of CBF-C that are necessary for formation of the CBF-A–CBF-C heterodimer.** (i) Analysis of heterodimer formation by several in vitro methods. To test the interactions of CBF-C with CBF-A, the mutant GST–CBF-C fusion polypeptides were incubated with radiolabeled CBF-A that was synthesized in a rabbit reticulocyte lysate and the association of GST–CBF-C and CBF-A, the mutant GST–CBF-C fusion polypeptides, with CBF-A was detected by the GST pull-down assay (Fig. 4A and Table 1). Like wild-type CBF-C, the deletion mutants which bound to DNA associated with CBF-A (data not shown). Mutant Δ42-45, which bound weakly to DNA, and mutants Δ47-52, Δ55-58, and Δ109-113, which did not bind to DNA, were able to interact with CBF-A in this assay (Fig. 4A, lanes 3 to 5 and 11). However, mutant Δ85-90, which bound very weakly to DNA, and mutants Δ61-65, Δ68-74, Δ92-95, and Δ100-104, which did not bind DNA, were unable to associate with CBF-A (Fig. 4A, lanes 6 to 10). This experiment indicates that residues within the segment of

| Deletion mutants of CBF-C | DNA Binding |
|---------------------------|------------|
| 1-124                     | +          |
| 1-175                     | +          |
| 1-119                     | +          |
| 1-110                     | -          |
| 40-119                    | -          |
| 34-175                    | -          |
| Δ42-45                    | ±          |
| Δ47-52                    | -          |
| Δ55-58                    | -          |
| Δ61-65                    | -          |
| Δ68-74                    | -          |
| Δ85-90                    | ±          |
| Δ92-95                    | -          |
| Δ100-104                  | -          |
| Δ109-113                  | -          |
| Δ116-120                  | -          |
CBF-A–CBF-C complex also coimmunoprecipitated after the mixture of wild-type CBF-C and CBF-A was cross-linked and then immunoprecipitated with the anti-CBF-A antibody (Fig. 4B, lane 4). All three experiments indicate that CBF-A interacted specifically with CBF-A to form a stable heterodimeric CBF-A–CBF-C complex.

On the basis of their interaction properties with CBF-A, the CBF-C mutants could be subdivided into three categories. Mutants Δ47-52, Δ55-58, and Δ109-113 behaved like wild-type CBF-C in that they formed cross-linked complexes with CBF-A (Fig. 4B, lanes 6, 10, and 34), were coimmunoprecipitated in the absence of cross-linking (lanes 7, 11, and 35), and were coimmunoprecipitated after cross-linking after cross-linking together with the cross-linked complex (lanes 8, 12, and 36). Mutant Δ68-74 did not form a cross-linked complex (lane 18), and did not coimmunoprecipitate with the anti-CBF-A antibody (lanes 19 and 20), indicating the mutant did not interact with CBF-A. In contrast, mutants Δ61-65, Δ85-90, Δ92-95, and Δ100-104 did not coimmunoprecipitate if not previously cross-linked (lanes 15, 23, 27, and 31), but were able to form a cross-linked complex (lanes 14, 22, 26, and 30); only the cross-linked complexes were coimmunoprecipitated (lanes 16, 24, 28, and 32). Interestingly, these four mutants did not associate with CBF-A in the GST pull-down assay. Formation of cross-linked complexes of these mutants with CBF-A indicates that these mutants interact with CBF-A to form a heterodimeric complex. But without cross-linking the complexes could not be isolated either by immunoprecipitation or by the pull-down method, both of which separate the dimeric complexes from the equilibrium state. This suggested that, unlike wild-type CBF-C, these mutants do not associate stably with CBF-A.

(ii) Analysis of the interaction of CBF-C with CBF-A using the in vivo yeast two-hybrid method. Recently, we demonstrated that CBF-A also interacts specifically with CBF-C in vivo using the yeast two-hybrid method with *S. cerevisiae* (26). We used this method to analyze the interactions of the mutants of CBF-C with CBF-A. The segment of CBF-C from residues 27 to 123 was cloned in frame downstream of the GAL4 DNA-binding domain (GAL4DB). A similar segment of each of the mutants was also cloned in frame with GAL4DB. The control segment of GAL4DB from residues 44 to 151, which is needed for formation of the CBF-DNA complex in the presence of CBF-B and CBF-C, was cloned in frame downstream of the GAL4 activation domain (GAL4AD). The GAL4DB–CBF-C plasmid was transformed together with the GAL4AD–CBF-A plasmid 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 (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining.

Colonies containing either GAL4DB–CBF-C or GAL4AD–CBF-A were unable to activate *lacZ* transcription. In contrast, colonies containing the combination of GAL4DB–CBF-C and GAL4AD–CBF-A stained blue with X-Gal, indicating that CBF-C interacted with CBF-A in vivo in *S. cerevisiae*, forming a GAL4DB–CBF-C–GAL4AD–CBF-A complex that activated *lacZ* transcription. Like wild-type CBF-C, mutants Δ47-52, Δ55-58, Δ61-65, Δ92-95, Δ100-104, and Δ109-113 developed blue colonies (Table 2), indicating that these mutants associate with CBF-A in vivo. Mutant Δ68-74, however, did not develop blue colonies, indicating that this mutant did not interact with CBF-A in vivo. Therefore, this mutant, which did not interact with CBF-A in any of our in vitro assays, also did not associate with CBF-A in vivo. In contrast, the mutants which did not associate with CBF-A by either the pull-down or coimmunoprecipitation assay but which did form a cross-linked complex...
with CBF-A interacted with CBF-A in vivo. Hence, the analysis of CBF-C mutants indicates that there are two classes of interactions between CBF-A and CBF-C: those which are indispensable for formation of a heterodimer and those that presumably stabilize the dimer. These two classes of interactions also correspond to different domains in CBF-C.

The interaction of CBF-A with the amino acid substitution CBF-C mutants which did not bind DNA were analyzed by the in vitro GST pull-down assay. The mutants were also fused with GAL4DB in the yeast plasmid to analyze their interactions by the in vivo yeast two-hybrid method. The results are summarized in Table 3. Mutants A46D/R47P, K49D, K50P, I51D, and F113R/I115K interacted with CBF-A in both the pull-down and the two-hybrid assay, as wild-type CBF-C did. In contrast, mutants L68E, A70P, and I101D/A104/E did not interact with CBF-A in the pull-down assay, but did interact with

FIG. 4. Interaction of the CBF-C mutants with CBF-A. The interaction of CBF-C with CBF-A was monitored in several ways. (A) GST pull-down assay. In vitro-translated labeled CBF-A (2 µl) was incubated with 500 ng of GST (lane 12), C1-175 (designated the wild type [wt] in lane 2), or the internal deletion mutants (lanes 3 to 11) as indicated. Lane 1 shows the input of the labeled CBF-A used in each reaction. (B) Cross-linking and communoprecipitation assays. Wild-type (lanes 1 to 4) and the internal deletion mutants of CBF-C (lanes 5 to 36) were cotranslated with CBF-A in vitro and were labeled. Each of the cotranslated labeled products (2 µl) was cross-linked with 2 mg of DMS per ml (lanes b) or was communoprecipitated with the anti-CBF-A antibody (lanes c), or was cross-linked first and then communoprecipitated (lanes d). The input of each cotranslated product is shown in lanes a. Bands of CBF-A, CBF-C, and cross-linked CBF-A-CBF-C (CBF-A/C) are indicated by arrowheads.

TABLE 1. Interactions of internal deletion mutants of CBF-C with CBF-A and CBF-B

| Internal deletion mutant | Interaction of CBF-C in the indicated assay with*:
|--------------------------|--------------------------------------------------|
|                          | CBF-A Pull-down | L.P. X-linking | CBF-B Pull-down | L.P. X-linking |
| 1-175 (wt)               | +              | +              | +               | +              |
| Δ42-45                   | +              | ND             | ND              | -              |
| Δ47-52                   | +              | +              | +               | +              |
| Δ55-58                   | +              | +              | -               | -              |
| Δ61-65                   | -              | -              | +               | -              |
| Δ68-74                   | -              | -              | -               | -              |
| Δ85-90                   | -              | -              | +               | -              |
| Δ92-95                   | -              | -              | -               | -              |
| Δ100-104                 | -              | -              | -               | -              |
| Δ109-113                 | +              | +              | +               | +              |

* The GST pull-down assay (pull-down), immunoprecipitation (L.P.), and cross-linking assay (X-linking) were done as described in the legends to Fig. 4 and 5. Interactions with CBF-A and CBF-B are classified as the same as the wild-type (wt) interaction (+), no interaction (−), or a weak interaction (±).

TABLE 2. Results of a yeast two-hybrid assay to determine in vivo interactions of CBF-C deletion mutants with CBF-A and CBF-B

| GAL4DB–CBF-C mutant* | Result of X-Gal staining of:
|-----------------------|-------------------------|
|                       | GAL4AD–CBF-B            |
| Wild type             | +                       |
| Δ47-52                | +                       |
| Δ55-58                | +                       |
| Δ61-65                | +                       |
| Δ68-74                | -                       |
| Δ92-95                | +                       |
| Δ100-104              | +                       |
| Δ109-113              | -                       |

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

+ X-Gal staining: +, blue color of same intensity as that of the wild-type CBF-C observed after 2 h; −, white color after 48 h.
CBF-A by the yeast two-hybrid method, suggesting that the mutants formed an unstable CBF-A–CBF-C complex in vitro. Mutant I77D/L80E did not interact with CBF-A by either the pull-down assay or the yeast two-hybrid method.

We conclude from the experiment that a relatively small segment of CBF-C, from residues 71 to 84, is dispensable for interaction with CBF-A and that a larger segment, from residues 59 to 104, is necessary for stable association between CBF-C and CBF-A.

Domains of CBF-C that are necessary for interaction of the CBF-A–CBF-C heterodimer with CBF-B. (i) Analysis of the interaction using in vitro methods. Our evidence indicated that CBF-B associated specifically with the CBF-A–CBF-C heterodimer and made contact with both CBF-A and CBF-C. To determine which part of CBF-C is necessary for association with CBF-B, we tested the CBF-C mutants for their ability to interact with CBF-B. The mutant GST–CBF-C polypeptides were first mixed with recombinant wild-type CBF-A and then incubated with in vitro-translated radiolabeled CBF-B, and their associations with CBF-B were detected by the GST pull-down assay (Fig. 5A and Table 1). Wild-type CBF-C associated with CBF-B (Fig. 5A, lane 2); one corresponding to the CBF-A–CBF-C complex and the other three, X, Y, and Z, corresponding to the three cross-linked complexes that CBF-B formed with CBF-A, with CBF-C, and with both CBF-A and CBF-C. The labeled CBF-A and CBF-C were not coimmunoprecipitated with the anti-CBF-B antibody in the absence of CBF-B with or without cross-linking (data not shown); however, both labeled subunits were coimmunoprecipitated in the presence of CBF-B before cross-linking (Fig. 5B, lane 3) and after cross-linking (lane 4). All four cross-linked products were also coimmunoprecipitated together with the un-cross-linked subunits (lane 4). These results indicate a stable association of CBF-B with the CBF-A–CBF-C heterodimer. Mutants Δ47-52, Δ55-58, Δ61-65, Δ100-104, and Δ109-113, which formed a cross-linked CBF-A–CBF-C complex, did not form the three X, Y, and Z cross-linked complexes (Fig. 5B, lanes 6, 10, 14, 30, and 34) and did not coimmunoprecipitate before or after cross-linking (lanes 7, 8, 11, 12, 15, 16, 31, 32, 35, and 36), indicating that these mutants did not interact with CBF-B. Mutant Δ68-74, which did not interact with CBF-A, also did not interact with CBF-B (lanes 18 to 20). In contrast, mutants Δ85-90 and Δ92-95 did not coimmunoprecipitate with the anti-CBF-B antibody without cross-linking (lanes 23 and 27) but formed the cross-linked X, Y, and Z complexes as well as the cross-linked CBF-A–CBF-C complex, all of which coimmunoprecipitated. Small amounts of the un-cross-linked subunits also coimmunoprecipitated in these reactions (lanes 24 and 28). This result indicates that these mutants interacted with CBF-B, but unlike wild-type CBF-C, they did not associate stably with CBF-B in a fashion similar to the way these mutants interacted with CBF-A.

The interactions of the amino acid substitution mutants of CBF-C with CBF-B were determined by the pull-down assay in the presence of CBF-A. The results are summarized in Table 3. Mutant A46D/R47P, which interacted with CBF-A, associated with CBF-B, like wild-type CBF-C. In contrast, mutants K94D, K50P, I51D, and F113R/I115K, which interacted with CBF-A, did not form a complex with CBF-B, indicating that these mutants were specifically defective in interacting with CBF-B. Mutant I77D/L80E, L68E, A70P, and I101D/A104E, which did not form a complex with CBF-A in the pull-down assay, also did not interact with CBF-B. The interactions of mutant A46D/R47P with both CBF-A and CBF-B were also tested by the cross-linking and the coimmunoprecipitation methods. The mutant also formed the three cross-linked X, Y, and Z complexes and the cross-linked CBF-A–CBF-C complexes, all of which coimmunoprecipitated, like wild-type CBF-C (data not shown). Thus, this mutant, although capable of forming the heterotrimeric CBF complex, was unable to bind to DNA.

(ii) Analysis of the interaction of CBF-C with CBF-B using the in vivo yeast two-hybrid method. Recently, using the yeast two-hybrid method, we showed that CBF-A interacted with CBF-B in vivo; our results implied that HAP5, the yeast homolog of CBF-C, mediated the in vivo interaction between CBF-A and CBF-B (26). We tested the interaction of CBF-C with CBF-B using the yeast two-hybrid method. 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-C. Yeast colonies containing only GAL4AD–CBF-B plasmid were X-Gal negative (data not shown), whereas yeast colonies containing both GAL4AD–CBF-B and GAL4DB–CBF-C stained blue with X-Gal (Table 2). We interpret this result as indicating that CBF-C interacts with CBF-B in vivo and that HAP3, the yeast homolog of CBF-A, mediated the interaction between CBF-C and CBF-B.

### Table 3. Properties of amino acid substitution mutants of CBF-C

| CBF-C mutanta | DNA binding | Interaction of CBF-C in the indicated assay withb |
|---------------|-------------|---------------------------------------------------|
|               |             | CBF-A | CBF-B |
|               |             | Pull-down | Two-hybrid | Pull-down | Two-hybrid |
| Wild type     | +           | +      | +          | +         | +         |
| A46D/R47P     | -           | -      | -          | -         | -         |
| K94D          | +           | +      | +          | +         | +         |
| K50P          | -           | -      | -          | -         | -         |
| I51D          | +           | +      | +          | +         | +         |
| L68E          | -           | -      | -          | -         | -         |
| A70P          | -           | -      | -          | -         | -         |
| I77D/L80E     | -           | -      | -          | -         | -         |
| I101D/A104E   | -           | -      | -          | -         | -         |
| F113R/I115K   | -           | -      | -          | -         | -         |

*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-A and CBF-B as shown in Fig. 3. The interactions of these mutants with CBF-A and CBF-B were examined by a GST pull-down assay as described in the legends to Fig. 4A and 5A and by the yeast two-hybrid assay described for Table 2.

*b* The nomenclature for each mutant specifies 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 two amino acids were substituted.

DNA binding is indicated as binding similar to that of wild-type CBF-C (+) or no binding (−).

Interactions with CBF-A and CBF-B are indicated as wild-type interactions (+) or no interactions (−).

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vol. 16, 1996 domains of cbf-c and interactions of cbf subunits 4009
We also used this method to analyze the interaction of the mutants of CBF-C with CBF-B (Tables 2 and 3). Mutants Δ68-74 and I77D/L80E, which did not interact with CBF-A in vitro or in vivo, did not develop blue yeast colonies, indicating that the mutants were defective in their interactions with CBF-B, because they were unable to recruit HAP3. Mutants Δ61-65, Δ92-95, Δ100-104, L68E, A70P, and I101D/A104E, which interacted with CBF-A in vivo but did not form stable complexes with CBF-A in vitro, did not develop blue colonies. This result suggests that these mutants did not recruit HAP3 stably and as a result did not interact with CBF-B in vivo. It is also possible that these mutants were specifically defective in interacting with CBF-B. Mutants Δ47-52, Δ55-58, Δ109-113, K49D, K50D, I51D, and F113R/I115K, which interacted with CBF-A in vitro and in vivo, did not develop blue colonies, indicating that these mutants specifically did not interact with CBF-B. In contrast, mutant A46D/R47P, which interacted with CBF-A in vitro and in vivo, developed blue yeast colonies, indicating that it did interact with CBF-B in vivo. This mutant also interacted with CBF-B in vitro in the pull-down assay. Thus, the CBF-C mutants which did not interact with CBF-B in vitro by the pull-down assay also did not interact with CBF-B in vivo in S. cerevisiae.

Therefore, we conclude that two segments of CBF-C, from residues 42 to 60, except residues 46 and 47, and from residues 105 to 113, are necessary for interactions of CBF-B with the CBF-A–CBF-C heterodimer, whereas residues 46 and 47 are necessary for formation of a complex between the heterotrimeric CBF and DNA.

The different interaction domains of CBF-C and their positions with respect to the histone-fold motif. In summary, our mutational analysis demonstrated that the part of CBF-C which is necessary for DNA binding consisted of two functional subunit interaction domains, one necessary for interaction with CBF-A to form the CBF-A–CBF-C heterodimer and the other necessary for interaction with CBF-B to form the heterotrimeric CBF (Fig. 6). Whereas a core part of CBF-C was sufficient for interaction with CBF-A in some assays, a larger portion was necessary to form a stable CBF-A–CBF-C complex. Two portions of CBF-C, one located at the N terminus and one at the C terminus of the CBF-A interaction domain, were essential for interaction of the CBF-A–CBF-C heterodimer with CBF-B. Mutational analysis also identified a DNA-binding domain which overlapped the N-terminal CBF-B interaction domain.

The amino acid sequences of the different domains of CBF-C are evolutionarily conserved and are similar to the sequence of the histone-fold motif of histone H2A. Crystallographic studies of the core histone octamer indicated that all four core histones, H2A, H2B, H3, and H4, contain a common three-dimensional structural motif, the histone fold, although the primary sequences of these histones showed only a low degree of homology (1). The histone fold consists of a short helix, followed by a short loop and β strand, a long helix,
another short loop and β strand, and another short helix; the overall structure appears to be a tandem duplication of two similar and contiguous helix-strand-helix motifs (2). Recently, Arents and Moudrianakis (3) reported the alignment of the amino acid sequences of the four histone folds on the basis of their three-dimensional structure and have classified these as (i) surface residues in the H2A-H2B dimer that are either exposed to the solvent or are in contact with DNA in the histone-octamer DNA complex; (ii) self residues that are involved in contacts within the H2A sequence; and (iii) pair residues that make contact with histone H2B to form the H2A-H2B dimer. The amino acids corresponding to surface, self, and pair residues of H2A and H2B showed that the surface, self, and pair residues of H2B are 53, 66, and 52% similar to the corresponding regions of CBF-A, respectively.

DISCUSSION

Our cross-link studies of the interaction of CBF-B with the CBF-A–CBF-C heterodimer provide clear evidence that CBF-B makes contact simultaneously with both CBF-A and CBF-C to form the heterotrimeric CBF-B(CBF-A–CBF-C) complex. As CBF-B is unable to interact separately with either CBF-A or CBF-C, it is likely that the heterodimerization of CBF-A with CBF-C generates a hybrid surface or interface which associates with CBF-B.

Our mutational analysis of CBF-C has defined the functional domains which are necessary for formation of the CBF-A–CBF-C heterodimer and for interaction of the heterodimer with CBF-B to form the CBF heterotrimer. Recently, we also identified two similar functional domains in the CBF-A subunit. Interestingly, the segments of both CBF-A and CBF-C that are needed for these interactions are evolutionarily conserved, are similar to each other, and are similar to the histone-fold motifs of histones H2B and H2A, respectively (Fig. 7) (26). This suggests that the three-dimensional structures of these portions of CBF-A and CBF-C are similar to each other and similar to the structure of the histone-fold motif. A recent structural analysis of the histone octamer indicated that the amino acid residues that are involved in the interaction between H2A and H2B are located primarily in helix II, the second loop and strand, and helix III of the histone folds (3) (see the pair residues of histone H2A in Fig. 6). This corresponds to the segment of CBF-C that provides the majority of interactions with CBF-A. Our results also suggest the existence of two qualitatively different degrees of interaction between CBF-C and CBF-A, corresponding to different segments of the histone-fold motif in CBF-C. Only a small part of the histone-fold motif, one that corresponds to only part of helix II, is necessary for interactions with CBF-A, but a larger portion, including the first strand, helix II, the second loop and strand, and part of helix III, is essential to form a stable CBF-A–CBF-C heterodimer (Fig. 6). However, in CBF-A the entire histone fold appears to be required for interaction with CBF-C. It is possible that a central portion in each of the heterodimeric domains of CBF-A and CBF-C makes primary contacts, whereas a larger portion may contribute additional contacts to form a stable CBF-A–CBF-C heterodimer or to maintain the structure of the domains.

Interestingly, three separate domains in the CBF-A–CBF-C heterodimer, one domain in CBF-A and the other two in CBF-C, are essential for interaction with CBF-B. These domains are located within the histone-fold motifs of the subunits. However, it is not clear how these three separate domains together form a surface which interacts with CBF-B. Our previous studies identified a subunit interaction domain in CBF-B consisting of 21 amino acid residues. From the evidence presented in this study, it is clear that the subunit interaction domain of CBF-B associates simultaneously with CBF-A and CBF-C to form the CBF heterotrimer. Thus, we speculate that the CBF-A–CBF-C heterodimer forms a tridentate contact surface with the subunit interaction domain of CBF-B in order to form the heterotrimeric complex.

Our study also showed that a small part of CBF-C, one that overlapped with one of the CBF-B interaction domains, is necessary for formation of the CBF-DNA complex. In our previous studies, we also identified a part of CBF-A that was necessary for the DNA binding of CBF. This portion of CBF-A also partially overlapped with the domain necessary for forma-
tion of the heterodimer. It is likely that we did not identify all the amino acid residues necessary for DNA binding, since some of these may overlap with those of the subunit interaction domains; indeed, the disruption of the subunit interactions also resulted in loss of DNA binding. Thus, more amino acid residues located within the subunit interaction domains of both CBF-A and CBF-C might be involved in formation of the CBF-DNA complex. Analysis of the structure of the histone octamer-DNA complex indicated that amino acid residues which bind DNA are located in several areas of the histone fold, the two loops and β strands, and the amino terminus of helix I. Previously, we identified a modular DNA-binding domain in the CBF-B subunit. Detailed structural studies of the CBF-DNA complex are needed to determine how the CBF subunits together form a complex with DNA. However, this study together with our previous studies indicated that amino acid residues present in all three CBF subunits are involved in formation of the CBF-DNA complex.

In conclusion, we have shown that the histone-fold motifs of CBF-A and CBF-C interact with each other and then with CBF-B to form the CBF heterotrimer, which in turn binds to a specific DNA sequence. In the core histones, the histone-fold motif provides the basis for histone dimerization, for formation of the histone octamer, and for binding of octamer to DNA in a sequence-independent fashion (3). In the CBF protein, the histone-fold motifs of CBF-A and CBF-C are involved in formation of the CBF-A–CBF-C heterodimer, but the CBF-A–CBF-C heterodimer interacts with CBF-B, which does not contain the histone-fold motif. Recently, histone-fold motifs were also found in the amino acid sequences of a large set of proteins with various functions in organisms ranging from archaebacteria to mammals, including two subunits of the drosophila TATA-binding-protein-associated factors (4). The role of the histone-fold motifs of these proteins remains to be determined. Since the histone-fold motif provides the basis for protein dimerization and oligomerization, it will be interesting to test whether the histone-fold motifs of CBF interact with nucleosomes or with other nuclear proteins containing a similar motif implicated in transcriptional regulation.

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