Pathway of Complex Formation between DNA and Three Subunits of CBF/NF-Y

PHOTOCROSS-LINKING ANALYSIS OF DNA-PROTEIN INTERACTION AND CHARACTERIZATION OF EQUILIBRIUM STEPS OF SUBUNIT INTERACTION AND DNA BINDING*

(Received for publication, July 17, 1998, and in revised form, August 28, 1998)

Shu Guang Liang and Sankar N. Maity‡

From the Department of Molecular Genetics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

In this study, we used a photocross-linking method to identify specific contact of CCAAT-binding factor (CBF) subunits in a CBF-DNA complex. The analysis showed that all three subunits in the CBF-DNA complex were cross-linked to DNA and that CBF-B and CBF-C were cross-linked more strongly than CBF-A. None of the CBF-A and CBF-C subunits, which together formed a CBF-A/CBF-C heterodimer, were cross-linked without CBF-B; in contrast, CBF-B was cross-linked in the absence of CBF-A/CBF-C. No subunit of heterotrimeric CBF containing DNA-binding domain mutant of either CBF-B or CBF-C was cross-linked to DNA, and interestingly, cross-linking of CBF-B that occurred without CBF-A/CBF-C was inhibited in presence of mutant CBF-C/CBF-A heterodimer. Altogether, these results indicated that the specific DNA contact surface of each CBF subunit is generated as a result of interaction between CBF-B and CBF-A/CBF-C heterodimer and that the three CBF subunits interact interdependently with DNA to form a CBF-DNA complex. Equilibrium interactions among the three CBF subunits and between CBF subunits and DNA were studied by electrophoretic mobility shift assay. This showed that at equilibrium DNA-binding conditions, the CBF-A/CBF-C heterodimer is very stable, but association between CBF-B and CBF-A/CBF-C is very weak. The nature of the association of CBF-B with CBF-A/CBF-C was also revealed by studying the inhibition of CBF-DNA complex formation by the mutant CBF-B. This study indicated that the association between CBF-B and CBF-A/CBF-C is stabilized upon interaction with DNA, a process likely to favor formation of a high-affinity CBF-DNA complex.

The CCAAT-binding protein CBF/NF-Y (referred to herein as CBF) binds specifically to DNA containing the CCAAT motif that is present in the proximal promoter of numerous mammalian genes (1–5). CBF/NF-Y consists of three subunits, CBF-A, CBF-B, and CBF-C, that all are required for DNA binding. In each CBF subunit, the segment needed for DNA binding is conserved from yeast to human, and interestingly, the conserved segments of CBF-A and CBF-C have shown homology with the histone-fold motifs of eukaryotic histones and archaeobacterial histone-like protein Hmf-2 (6–8).

Previous mutational analyses have shown that the conserved segment of both CBF-A and CBF-C consist of overlapping domains of subunit interactions and DNA binding (9, 10) and that the conserved segment of CBF-B, in contrast, contains two separate domains of subunit interaction and DNA binding (11). According to this analysis, large portions of the histone-fold motifs of CBF-A and CBF-C interact with each other to form a CBF-A/CBF-C heterodimer, and within the heterodimer, the two subunits together generate a surface that interacts with CBF-B. Mutations of CBF-A and CBF-C that inhibit formation of the heterodimer or the heterotrimer also inhibit formation of the CBF-DNA complex. Similarly, mutation of CBF-B that does not interact with the CBF-A/CBF-C heterodimer is unable to bind to DNA. These results indicated that the interactions among the CBF subunits are required for DNA binding. The DNA binding domain of each CBF subunit was defined based on mutation in this domain that specifically inhibited DNA binding but not formation of the heterotrimeric CBF. Identification of the DNA-binding domain in each CBF subunit suggested that all three CBF subunits in the CBF-DNA complex interact with DNA.

Recently, the interaction between CBF and DNA was analyzed using hydroxyl radical footprinting and the methylation interference assay. In the CBF-DNA complex, three separate DNA regions located within a length of 30 base pairs are protected from hydroxyl radical cleavage. However, methylation of bases in only the middle protected region that contain the CCAAT sequence interfered with CBF binding. The specific DNA sequence required for CBF binding was determined by using the polymerase chain reaction-mediated random binding site selection method. This study showed that in addition to the CCAAT sequence, specific flanking sequences located at both ends of the CCAAT are required for CBF binding and that all of these specific sequences are located within one footprinting region containing the CCAAT sequence (12). Together, these data indicated that the CBF contact over the CCAAT motif region is sequence-specific, whereas the contacts at both sides of this region are not. Which CBF subunit makes contact in each of the protected regions remains to be determined.

To obtain clearer insight into the interaction between CBF and DNA, we determined specific contacts of CBF subunits in the CBF-DNA complex with the photocross-linking method. To evaluate the pathway for DNA binding of the CBF subunits, we

* This work was supported by Grant AR43660 (to S. N. M.) from the National Institutes of Health. DNA sequencing of plasmid constructs was performed by The University of Texas M. D. Anderson Cancer Center Core Sequencing Facility, which is supported by NCI, National Institutes of Health Grant CA16672. The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. of Molecular Genetics, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Tel.: 713-792-8943; Fax: 713-794-4295; E-mail: sankar.maity@mdgen.mdacc.tmc.edu.

1 The abbreviations used are: CBF, CCAAT-binding factor; EMSA, electrophoretic mobility shift assay; BrdUrd, bromodeoxyuridine; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; HMK, heart muscle protein kinase.

This paper is available on line at http://www.jbc.org
studied equilibrium interactions between CBF subunits and between CBF and DNA with the electrophoretic mobility shift assay (EMSA).

MATERIALS AND METHODS

Generation and Purification of Recombinant CBF Subunits—Full-length CBF-A and CBF-B were cloned into the NdeI and XhoI sites of the pET-23b vector (Novagen Inc., Madison, WI), and each subunit was expressed in BL21(DE3)pLysS Escherichia coli strain as a fusion with a His$_{6}$ tag at the C-terminal end of the subunit. The full-length and a truncated form (C1–175) of CBP-C were expressed in E. coli as a fusion with glutathione S-transferase (GST) as described previously (10). Expression of each subunit was induced by 1 mM isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) for 3 h at the exponential phase of bacterial growth. After induction, the cell suspensions were centrifuged, and the bacterial pellets were resuspended with 6 mM urea in Buffer I containing 50 mM Tris-HCl (pH 7.9), 50 mM Na$_{2}$PO$_{4}$, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol and lysed by sonication.

The bacterial extract containing recombinant CBF-B was fractionated to purify the CBF-B subunit, whereas the extracts containing recombinant CBF-A and recombinant CBF-C were mixed and fractionated together to purify the CBF-A/CBF-C heterodimer. The extract containing CBF-B was first loaded into a nickel-nitrotriacetic acid-agarose affinity column (Qiagen Inc., Valencia, CA) and then washed extensively with Buffer I containing 6 M urea; the bound protein was renatured by washing in several steps with Buffer I containing a decreasing concentration of urea from 6 to 0 M. The renatured CBF-B was eluted from the resin with 0.5 M imidazole in Buffer I. The eluate was dialyzed for 3 h against Buffer II containing 25 mM HEPES, pH 7.9, 1 mM dithiothreitol, and 100 mM NaCl, and the dialysate was loaded into a MonoS cation exchange column. The column was washed with Buffer II, and the CBF-B polypeptide was eluted from the column by a linear salt gradient. The peak activity of CBF-B was eluted at 400 mM NaCl.

Equal volumes of bacterial extracts containing CBF-A and GST-CBF-C were mixed, and the proteins of the extract were renatured by dialysis against Buffer III containing 50 mM Tris-HCl, 75 mM KCl, 10% glycerol, 1 mM EDTA, 0.05% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. Both polypeptides, which were incubated at room temperature for 10 min in a buffer used in the DNA binding reaction containing 25 mM HEPES, pH 8.0, 75 mM KCl, 10% glycerol, 1 mM EDTA, 0.05% Nonidet P-40, 0.5 mM dithiothreitol, and 100 mM NaCl, and the dialysate was loaded into a MonoS cation exchange column. The column was washed with Buffer III, and the CBF-B polypeptide was eluted from the column by a linear salt gradient. The peak activity of CBF-B was eluted at 400 mM NaCl. 

DNA Binding and Subunit Interactions—DNA binding of recombinant CBF subunits was performed in a reaction containing labeled DNA of o2(1) collagen promoter as described before (6). The association between CBF subunits was done using both labeled and unlabeled CBF polypeptides, which were incubated at room temperature for 10 min in a buffer used in the DNA binding reaction containing 25 mM HEPES, pH 8.0, 75 mM KCl, 10% glycerol, 1 mM EDTA, 0.05% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. Both DNA binding and subunit interaction reactions were fractionated on a 4% polyacrylamide gel in a buffer containing 50 mM Tris, 380 mM glycine, and 4.5 mM EDTA, and the labeled components were detected by autoradiography.

RESULTS

Photocross-linking of CBF-DNA Complex—Previously, hydroxyl radical footprinting analysis of CBF-DNA complex has shown that three separate DNA regions are protected from hydroxyl radical cleavage (12) (Fig. 1A). To determine specific contacts of CBF subunits in the protected regions, we adopted a photocross-linking method using BrdUrd-substituted DNA of the o2(1) collagen promoter containing the CBF binding site. Substitution of BrdUrd residue was introduced at different positions of both upper and lower parts of the double-stranded oligonucleotides shown in Fig. 1B. The substitution of BrdUrd residue in these DNA positions did not affect the formation of the CBF-DNA complex, which was examined by EMSA.

CBF-C was labeled by phosphorylation with bovine muscle kinase and [$\gamma$-32P]ATP in HMK reaction buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 12 mM MgCl$_{2}$. Labeled protein was separated from free labeled ATP by a Micro Bio-Spin Chromatography Column (Bio-Rad).

Bromodeoxyuridine (BrdUrd)-substituted Double-stranded Oligonucleotides—The following oligonucleotides were synthesized by Genosys from the nucleotide sequence of the CBF binding site of the o2(1) collagen promoter: a1 (5'-CCCGGGCCCTAGCTGCTAGCTTATTGGTGGAGGACCTTTTTGGAAG-3'), a2 (5'-CCCGGGCCCTAGCTGCTAGCTTATTGGTGGAGGACCTTTTTGGAAG-3'), a3 (5'-CCGGGGCCCTAGCTGCTAGCTTATTGGTGGAGGACCTTTTTGGAAG-3'), a4 (5'-CCGGGGCCCTAGCTGCTAGCTTATTGGTGGAGGACCTTTTTGGAAG-3'), a5 (5'-CCGGGGCCCTAGCTGCTAGCTTATTGGTGGAGGACCTTTTTGGAAG-3'), a6 (5'-CCGGGGCCCTAGCTGCTAGCTTATTGGTGGAGGACCTTTTTGGAAG-3'), b1 (5'-CTCAAAAAAGTCTCCACCAATTTGAGGAGTGGCTAGGCGGATAG-3'), b2 (5'-CTCAAAGAACGTCTCCACCAATTTGAGGAGTGGCTAGGCGGATAG-3'), b3 (5'-CTCAAAAAAGTCTCCACCAATTTGAGGAGTGGCTAGGCGGATAG-3'), b4 (5'-CTCAAAAAAGTCTCCACCAATTTGAGGAGTGGCTAGGCGGATAG-3'), b5 (5'-CTCAAAAAAGTCTCCACCAATTTGAGGAGTGGCTAGGCGGATAG-3'), b6 (5'-CTCAAAAAAGTCTCCACCAATTTGAGGAGTGGCTAGGCGGATAG-3'). L1, L2, and mutL2 oligonucleotides were formed first, by hybridizing a1 with b2, b3, and mutb3, respectively; U1, U2, and U3 were formed by hybridizing b1 with a2, a3, and a4, respectively. Each hybridized oligonucleotide was then labeled and substituted with BrdUrd by fill-in reaction containing Klenow DNA polymerase, BrdUrdTP, and [$\gamma$-32P]dCTP. The L3 and L4 oligonucleotides were formed by hybridizing a1 with b4 and b5, respectively, and the U4 and U5 oligonucleotides were formed by hybridizing b1 with a5 and a6, respectively. Each oligonucleotide (b4, b5, a5, and a6) contained a single BrdUrd substitution that was incorporated during oligonucleotide synthesis. The L3, L4, U4, and U5 oligonucleotides were labeled by the fill-in reaction using [$\gamma$-32P]dCTP.

Analysis of Photocross-linking Reactions—The CBF subunits (60 ng of CBF-B and 80 ng of CBF-A/CBF-C) were first incubated in a 10-µl volume with 20 fmol of BrdUrd-substituted labeled DNA at room temperature for 20 min in the dark. The reaction was irradiated with a UV light source (Model UVS 28, two 8-W 254-nm bulbs, Ultraviolet Products Inc.) for 5 min at room temperature. After irradiation, the reaction was analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoprecipitated with anti-CBF antibodies and then analyzed by SDS-PAGE. The immunoprecipitation was performed as described previously (11) with the following modifications: after UV irradiation, one half of the reaction was incubated with 1 µl of antibody, and the other half was first mixed with an equal volume of radiolabeled precipitation buffer containing 150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 1.0% Triton X-100, and 0.5 M NaCl for 10 min at 4 °C, centrifuged to remove the antibody-antigen complexes were precipitated with protein A-Sepharose, and the precipitated material was analyzed by SDS-PAGE. The cross-linked labeled DNA-protein was detected in the gel by autoradiography.

CBF-C was labeled by phosphorylation with bovine muscle kinase and [$\gamma$-32P]ATP in HMK reaction buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 12 mM MgCl$_{2}$. Labeled protein was separated from free labeled ATP by a Micro Bio-Spin Chromatography Column (Bio-Rad).
Recombinant CBF subunits used in the DNA binding reactions were purified extensively from bacterial extracts; CBF-B was purified separately, whereas CBF-A and CBF-C were purified as CBF-A/CBF-C heterodimer (Fig. 2A). BrdUrd-substituted radiolabeled DNA was first incubated with the recombinant CBF subunits; then the reaction was irradiated with UV light and the photoreaction was analyzed by SDS-PAGE to detect a covalently cross-linked DNA-protein complex. Both U1 and L1 DNAs, which contained BrdUrd substitutions in the upper and lower strands, respectively, formed a cross-linked complex when the reaction contained only the CBF-B subunit (Fig. 2B, lanes 2 and 5). In contrast, none of the DNAs formed a cross-linked complex when the reaction contained only CBF-A/CBF-C heterodimer (Fig. 2B, lanes 3 and 6). When the reaction contained both CBF-B and CBF-A/CBF-C, the U1 DNA developed two cross-linked complexes of 60- and 32-kDa species, whereas the L1 DNA formed three cross-linked complexes of 60-, 40-, and 32-kDa species (Fig. 2B, lanes 4 and 7). Because the molecular weight of the cross-linked CBF subunits is different from that of the uncross-linked subunits, we utilized immunoprecipitation method using anti-CBF antibodies to detect CBF subunits in each of the cross-linked complexes. The irradiated reaction containing both CBF-B and CBF-A/CBF-C was immunoprecipitated with anti-CBF-A antibodies. In this reaction, all three cross-linked complexes of L1 DNA were immunoprecipitated together without heat treatment, indicating that the complexes were immunoprecipitated as a part of the CBF-DNA complex. Several other DNAs containing BrdUrd substitutions in only one hydroxyl radical footprinting region were used in the photocross-linking reactions. Locations of the BrdUrd substitutions in the nucleotide sequence are shown in Fig. 1B, and the results of photocross-linking reactions are shown in Fig. 4 and summarized in Fig. 5.
rized in Table I. L2 DNA containing BrdUrd substitutions in footprinting region I was cross-linked with all three CBF subunits, similar to L1 DNA (Fig. 4, lanes 1 and 2). U3 DNA, which contained BrdUrd substitutions in footprinting region III, was cross-linked with both CBF-B and CBF-C, similar to the U1 DNA (Fig. 4, lanes 3 and 5). U5 DNA, which contained only one BrdUrd substitution, located in the footprinting region I, was cross-linked with all three CBF subunits (Fig. 4, lane 6). These results indicated that CBF-B and CBF-C subunits make contact throughout all three protected DNA regions, whereas the CBF-A subunit makes contact in footprinting regions I and II.

To determine the specificity of the cross-linking reactions, DNA binding reactions were constituted with amino acid-substituted mutant CBF subunits and also with nucleotide-substituted mutant DNA that does not bind CBF. Previously, we have isolated the DNA-binding domain mutant of each CBF subunit that formed a heterotrimeric CBF but did not bind DNA. We tested each of mutant CBF-B and mutant CBF-C together with the other two wild-type subunits in the cross-linking reaction containing L1 DNA. Similar to wild-type CBF-B, mutant CBF-B was cross-linked to L1 DNA in the absence of CBF-A/CBF-C dimer (Fig. 5, lanes 2 and 5). However, when the reaction contained a mixture of mutant CBF-B and wild-type CBF-A/CBF-C dimer, none of the subunits was cross-linked to DNA (Fig. 5, lane 4). Similarly, no subunit was cross-linked when the reaction contained a mixture of wild-type CBF-B and mutant CBF-C/CBF-A dimer (Fig. 5, lane 3). The cross-linking of CBF-B to DNA, which occurred in the absence of CBF-A/CBF-C dimer, was likely due to nonspecific DNA contact of CBF-B, because mutation of CBF-B did not inhibit CBF-B cross-linking to DNA. Moreover, a mutant L1 DNA that did not bind CBF was also cross-linked to wild-type CBF-B in a reaction containing all three wild-type CBF subunits, but not in the reaction with mutant CBF-C, indicated that CBF-B makes specific contact with DNA in the CBF-DNA complex. These results showed that...
Photocross-linking reactions containing different wild-type and mutant DNA and of wild-type CBF subunits to mutant DNA. The photocross-linking reactions with L1 DNA (lanes 1–5) and with mutL1 DNA (lanes 6 and 7) as described in Fig. 2B. The cross-linking of CBF subunits with each DNA is summarized in Table I.

### Table I

Summary of photocross-linking reactions with α2(1) collagen promoter DNAs containing BrdUrd substitutions at different positions

| Oligonucleotide | Cross-linked subunits |
|-----------------|-----------------------|
| L1              | +                     |
| L2              | +                     |
| L3              | +/−                   |
| L4              | +/−                   |
| U1              | −                     |
| U2              | −                     |
| U3              | −                     |
| U4              | +                     |
| U5              | +                     |

*Positions of BrdUrd substitutions in these oligonucleotides are shown in Fig. 1.*

*The results of photocross-linking reactions with L1, L2, U1, U2, U3, and U5 are shown in Figs. 2B, 3A, and 4; data of the reactions with L3, L4, and U4 are not shown. The CBF subunits cross-linked to the individual DNA, are indicated by + (strongly cross-linked), +/− (weakly cross-linked), and − (not cross-linked).*

The formation of CBF-DNA complex at different subunit concentrations. Radiolabeled DNA of the α2(1) collagen promoter (1 nM) was incubated with different concentrations of purified recombinant CBF-B and CBF-A/CBF-C, and formation of CBF-DNA complex was detected by EMSA. Each lane contained equimolar amounts (in nM) of CBF-B and CBF-A/CBF-C; lane 1, 0.004 nM; lane 2, 0.02 nM; lane 3, 0.04 nM; lane 4, 0.1 nM; lane 5, 0.2 nM; lane 6, 0.4 nM; lane 7, 1 nM; lane 8, 2 nM.

Characterization of Equilibrium Steps of the DNA Binding Pathway—To better understand the DNA binding process, we used highly purified recombinant CBF subunits to quantitatively characterize subunit interactions and DNA binding. The association between the CBF subunits was analyzed by non-denaturing polyacrylamide gel electrophoresis, the EMSA method, which is normally employed to detect DNA binding.

The formation of CBF-DNA complex was determined using 1 nM of labeled DNA and increasing concentrations of both CBF-B and CBF-A/CBF-C (Fig. 6, lanes 1–8). In this experiment, 50% of the labeled DNA formed a CBF-DNA complex when the binding reaction contained 1 nM CBF-B and 1 nM CBF-A/CBF-C, and the apparent equilibrium constant of this reaction is $2.2 \times 10^9$. To detect subunit interactions by EMSA, we used radiolabeled recombinant CBF-C, which was synthesized in bacteria as fusion with the recognition site of HMK, purified as CBF-A/CBF-C heterodimer as shown in Fig. 2A, and radiolabeled by phosphorylation using HMK. The phosphorylated CBF-C/CBF-A heterodimer and CBF-B formed a CBF-DNA complex with the same affinity as the unphosphorylated protein (data not shown). When the radiolabeled heterodimer was analyzed by EMSA, it moved as a single labeled band (Fig. 7, lane 1). To detect the presence of unlabeled CBF-A in the labeled band, we first mixed the labeled heterodimer with anti-CBF-A antibodies and then analyzed by EMSA. This resulted in formation of a band with much slower mobility (Fig. 7A, lane 2), indicating that the labeled band is a CBF-A/CBF-C complex. Moreover, when a CBF-A/CBF-C heterodimer containing unlabeled CBF-C and labeled CBF-A (CBF-A was synthesized and labeled similarly to CBF-C) was separated by EMSA, the complex moved with the same mobility as the heterodimer containing labeled CBF-C (data not shown). These results demonstrated that the CBF-A/CBF-C complex moved in the nondenaturing gel as an undissociated heterodimer. When decreasing concentrations of heterodimer were separated in the gel, no change in mobility was observed, indicating that the heterodimer is stable (data not shown).

The heterodimer is resistant to denaturation in a 3 M concentration of urea, but it can be denatured completely in 6 M urea (Fig. 7A, lanes 3–7). In this regard, it is interesting to note that the histone H2A/H2B dimer, both of which contain histone-fold motifs, are also resistant to denaturation in 3.5 M urea.

![Fig. 4. Photocross-linking of CBF subunits with DNAs containing BrdUrd substitutions at different positions. Position of BrdUrd in each DNA are shown in Fig. 1B. Each radiolabeled DNA was incubated with both CBF-B and CBF-A/CBF-C, treated with UV light, and then analyzed by SDS-PAGE as described in Fig. 2B. The cross-linking of CBF subunits with each DNA is summarized in Table I.](image)

![Fig. 5. Photocross-linking of mutant (mut) CBF subunits to wild-type DNA and of wild-type CBF subunits to mutant DNA. The photocross-linking reactions containing different wild-type and mutant CBF subunits, indicated at top of each lane, were performed with L1 DNA (lanes 1–5) and with mutL1 DNA (lanes 6 and 7) as described in Fig. 2B. The mutL2, which contained a single nucleotide substitution in the L1 sequence, is shown in Fig. 1B.](image)

in the CBF-DNA complex, all three CBF subunits made specific contact with DNA and that mutation of one subunit resulted in inhibiting all contacts of the three subunits.
but were denatured completely in 5.5 M urea (13). The CBF-A/CBF-C heterodimer is, however, unstable in heat and was denatured completely at 65 °C (lanes 8–11), suggesting that it is likely to be a globular protein.

To detect an association of CBF-B with CBF-A/CBF-C, the radiolabeled heterodimer was mixed with purified recombinant CBF-B and analyzed by EMSA. Surprisingly, when a mixture containing 2 nM CBF-B and 2 nM CBF-A/CBF-C was separated, the mobility of the heterodimer was unchanged. Moreover, no significant change in its mobility was observed in a mixture containing 20 nM CBF-A/CBF-C and 20 nM CBF-B (data not shown). We have shown previously that the formation of heterotrimeric CBF was detected by cross-linking with glutaraldehyde, which cross-links both strongly and weakly interacting polypeptides and also cross-links multiple polypeptides in a higher-order complex. When a mixture containing 1 nM CBF-B and 1 nM CBF-A/CBF-C was cross-linked with 0.01% glutaraldehyde and then separated on SDS-polyacrylamide gel, a cross-linked complex corresponding to the heterotrimeric CBF was detected (data not shown). This indicated that, indeed, CBF-B and CBF-A/CBF-C formed a heterotrimeric complex but that, unlike the CBF-A/CBF-C heterodimer, the heterotrimeric complex was unstable or highly reversible.

**Inhibition of DNA Binding of Wild-type CBF by a Mutant CBF-B Subunit**—Previous studies have shown that a DNA-binding domain mutant of CBF-B, which interacts with CBF-A/CBF-C but does not bind with DNA, inhibits DNA binding of wild-type CBF subunits (14). The studies indicated that the mutant CBF-B acts as a competitive inhibitor of interaction between wild-type CBF-B and CBF-A/CBF-C, and forms an inactive complex with CBF-A/CBF-C. Our current results showed, however, that the interaction between wild-type CBF-B and CBF-A/CBF-C is not stable. Association of CBF-A/CBF-C with the mutant CBF-B was studied by the EMSA and by cross-linking with glutaraldehyde. The result was that, similar to the interaction of wild-type CBF-B, the association of mutant CBF-B with the CBF-A/CBF-C heterodimer was unstable.

To examine the effect of the mutant CBF-B in binding of wild-type CBF subunits, a 10-fold excess of the mutant CBF-B (20 nM) was added in a DNA binding reaction containing 2 nM wild-type CBF-B, 2 nM wild-type CBF-A/CBF-C, and 1 nM labeled DNA (Fig. 8A). In this reaction, no significant inhibition of wild-type CBF-DNA complex formation was observed (Fig. 8B, lanes 1 and 2). Similarly, no inhibition of DNA binding of wild-type CBF occurred even after the wild-type CBF-A/CBF-C was preincubated with the excess quantity of mutant CBF-B and then mixed with the wild-type CBF-B and labeled DNA (Fig. 8B, lane 4). The excess quantity of mutant CBF-B was totally inactive in DNA binding; no DNA-protein complex was detected when the reaction contained 20 nM mutant CBF-B, 2 nM wild-type CBF-A/CBF-C, and 1 nM labeled DNA (data not shown). These results indicated that the association of the mutant CBF-B with CBF-A/CBF-C, which would form an inactive complex, did not occur in the reactions in Fig. 8B, lanes 2 and 4. In contrast, when the excess quantity of CBF-B was added to a DNA binding reaction containing the wild-type CBF subunits, the labeled DNA, and 20 μg/ml of double-stranded poly(dI-dC) DNA, the formation of wild-type CBF-DNA complex was severely inhibited (Fig. 8C, lanes 1 and 2). When wild-type CBF-A/CBF-C was preincubated with mutant CBF-B in the presence of poly(dI-dC) and then mixed with wild-type CBF-B and labeled DNA, the formation of wild-type CBF-DNA complex was inhibited more (Fig. 8C, lane 4). But when the wild-type CBF-B was preincubated with CBF-A/CBF-C and the mutant CBF-B was added, formation of the CBF-DNA complex was less inhibited (Fig. 8C, lane 3). We interpreted these results as indicating that the mutant CBF-B associated with wild-type CBF-A/CBF-C in the presence of poly(dI-dC) and formed an inactive complex. However, when the wild-type CBF-DNA complex was first formed by preincubating wild-type CBF-B, wild-type CBF-A/CBF-C, labeled DNA, and poly(dI-dC)
and the excess quantity of mutant CBF-B was added, no inhibition of CBF-DNA complex formation occurred (Fig. 8C, lane 5). Thus, the mutant CBF-B specifically inhibited the association reaction between DNA and the wild-type CBF subunits but was unable to dissociate the preformed CBF-DNA complex, indicating that CBF-DNA is a very stable complex.

Because the poly(dI-dC) is usually added as a nonspecific DNA in a DNA binding reaction, we tested whether other nonspecific double-stranded DNAs, such as poly(dG-dC) and poly(dA-dT), affected the mutant CBF-B-mediated inhibition of specific DNA binding. Mutant CBF-B interfered strongly with DNA binding of wild-type CBF in the presence of 10 μg/ml poly(dG-dC), similar to poly(dI-dC) (Fig. 9B, lanes 1–10). In presence of 10 μg/ml poly(dA-dT), mutant CBF-B inhibited weakly, but at a higher concentration, it inhibited strongly (Fig. 9B, lanes 11–14). These results showed that the mutant CBF-B requires the presence of nonspecific DNA to inhibit specific DNA binding of wild-type CBF subunits. Because it is possible that nonspecific DNA facilitates interaction between mutant CBF-B and CBF-A/CFB-C, which do not form a stable complex at low subunit concentrations. We used the EMSA again to assay the interaction of labeled CBF-A/CFB-C with CBF-B in the presence of poly(dI-dC). As shown earlier, 10 nM of the labeled CBF-A/CFB-C did not form any complex with either 10 nM wild-type CBF-B or 10 nM mutant CBF-B (Fig. 10, lanes 1–3). The labeled CBF-A/CFB-C also did not form any complex with 50 μg/ml of poly(dI-dC) (Fig. 10, lane 4). In contrast, the labeled CBF-A/CFB-C formed a complex when poly(dI-dC) was present in a reaction containing either wild-type or mutant CBF-B (Fig. 10, lanes 5 and 6). The complex was not observed when the reaction contained a 50-fold lower concentration of poly(dI-dC) (data not shown). Thus, the complex was formed in the presence of high concentration poly(dI-dC), which is 1000-fold molar excess of specific DNA present in the DNA binding reaction, indicating that the complex is a low-affinity DNA-protein complex. Furthermore, this complex was formed only in the presence of both CBF-B and CBF-A/CFB-C, which together formed an unstable heterotrimer, indicating that poly(dI-dC) interacts specifically with the unstable heterotrimer and forms the low-affinity DNA-protein complex that appears more stable than the heterotrimer. The poly(dI-dC) also formed a low affinity DNA-protein complex equally with the heterotrimer containing mutant CBF-B, which did not form the specific DNA-protein complex. We concluded, therefore, that nonspecific DNA stabilized the interaction between mutant CBF-B and wild-type CBF-A/CFB-C by forming a low-affinity DNA-protein complex and that this resulted in inhibiting of specific DNA binding of wild-type CBF subunits in a reaction containing an excess of both mutant CBF-B and nonspecific DNA.

**DISCUSSION**

Our photocross-linking experiment showed that each of the three CBF subunits makes contact with DNA in the CBF-DNA complex. Previous hydroxyl radical footprinting of the CBF-DNA complex demonstrated that three DNA regions are protected from hydroxyl radical cleavage by the CBF contacts, suggesting the possibility that each of the three CBF subunits makes contact separately in the three protected DNA regions (12). To test this possibility, we performed the photocross-linking reaction using DNAs containing BrdUrd substitutions at different positions of the protected regions. This analysis showed the CBF-B and CBF-C subunits to be cross-linked irrespective of BrdUrd position in the DNA, indicating that the two subunits make contact in all three protected regions. In contrast, the CBF-A subunit was cross-linked to a limited number of DNAs with BrdUrd substitutions, located mainly in footprinting regions I and II, indicating that CBF-A makes contact in these two protected regions.

Previously, mutational analysis of CBF subunits demonstrated that each CBF subunit consists of a DNA-binding domain located in the conserved segment of each subunit (9–11). The conserved segment of CBF-B that is necessary for DNA binding consists of two modular domains, one for interaction with CBF-A/CFB-C and one for DNA binding. Unlike the modular arrangement of CBF-B, the conserved segments of CBF-A and CBF-C consist of overlapping subunit interaction and
DNA-binding domains. It is important to note that the DNA-binding domains are defined based on mutations that formed heterotrimeric CBF but did not bind to DNA, whereas the subunit interaction domains are defined by mutations that inhibited both subunit interaction and DNA binding. Because the DNA binding domains of CBF-A and CBF-C overlapped with the subunit interaction domains, it was not possible to identify the boundaries of these domains in the two subunits. In a complex protein like CBF, identification of the DNA-binding domain does not ensure that the domain interacts directly with DNA. Our analysis showed, however, that all three CBF subunits make contact with DNA. Thus, it is possible that the DNA-binding domain of each CBF subunit interacts directly with DNA. According to the mutation analysis, the DNA-binding domain of CBF-A, which was defined by seven different mutants, is much larger than the DNA-binding domain of CBF-C, which was defined by a single mutant. In contrast, the photocross-linking analysis showed that CBF-C makes contact with larger portion of DNA than does CBF-A, indicating that a larger, still unidentified portion of CBF-C, which is located within the subunit interaction domains, interacts with DNA.

Although both CBF-A and CBF-C consist of DNA-binding domains and were cross-linked to DNA in the CBF-DNA complex, none of the subunits were cross-linked to DNA in the absence of CBF-B, suggesting that in the heterodimer, the DNA contact surfaces of both CBF-A and CBF-C are inaccessibly not structured. Interestingly, both CBF-A and CBF-C showed homology with the histone-fold motifs of histones H2A, H2B, and archaebacterial Hmf-2, which interact with DNA sequence-independent manner. Recent studies of the high-resolution structure of nucleosomes revealed that the histone-fold consists of a short α-helix (helix I) followed by a loop and β-strand (L1), a long helix (helix II), another short loop and β-strand (L2), and a short helix (helix III) (15). In the heterodimeric histones, the histone-fold motifs associate with each other in an antiparallel fashion and create two types of DNA-binding surfaces, one provided by interaction of loop L1 of one histone with loop L2 of another, and the other by helix I of two histones. Two arginine residues present in L1 and L2 of histone H2A make contact with a minor groove of DNA in the histone octamer-DNA complex (16). These two arginines are not conserved in CBF-C, which showed homology with the histone H2A, suggesting that the mode of DNA binding of CBF-A/CBF-C in the CBF-DNA complex is different from that of the histone octamer-DNA complex.

Unlike the CBF-A/CFB-C heterodimer, the CBF-B subunit was cross-linked to DNA in the absence of CBF-A/CFB-C. However, CBF-B was also cross-linked to mutant DNA, which does not bind CBF. Moreover, a DNA-binding domain mutant CBF-B that formed heterotrimeric CBF but did not bind DNA was also cross-linked to the wild-type DNA. These results indicate that CBF-B made nonspecific contact with DNA, possibly because CBF-B is rich in basic amino acids that favor electrostatic interaction with DNA. Interestingly, the nonspecific DNA contact of CBF-B was inhibited in the presence of CBF-A/CFB-C, suggesting that interaction of CBF-B with CBF-A/CFB-C resulted in modulating CBF-B, probably masking the surface, which makes nonspecific contact with DNA. However, in the specific CBF-DNA complex, CBF-B made contact with DNA, indicating that interaction of CBF-B with CBF-A/CFB-C inhibited nonspecific DNA contact of CBF-B but facilitated specific DNA contact. Hence, it is reasonable to propose a model in which the interaction between CBF-B and CBF-A/CFB-C results in generating a surface in each CBF subunit that makes specific contact with DNA. None of the three CBF subunits in a mixture containing a DNA-binding domain mutant of either CBF-B or CBF-C was cross-linked to DNA, indicating that the DNA contacts of the CBF subunits are interdependent. It is possible that the three CBF subunits together generate a hybrid surface that interacts with DNA and that mutation of single subunit inhibits this interaction.

Our quantitative determination of subunit interactions and DNA binding by EMSA showed that the CBF-A/CFB-C heterodimer was very stable at equilibrium binding condition in which 50% or less CBF-DNA complex was formed. This stability may be the result of extensive interaction between the histone-fold motifs of both CBF-A and CBF-C. This is consistent with our previous mutational analysis, which showed that a large segment of CBF-A and large segment of CBF-C are required to form a heterodimeric CBF-A/CFB-C complex. Unlike the CBF-A/CFB-C heterodimer, the association of CBF-B with CBF-A/CFB-C, which formed heterotrimeric CBF, could not be detected by EMSA. However, interaction of CBF-B with CBF-A/CFB-C could be detected at a low subunit concentration by a cross-linking method using glutaraldehyde, which suggested that CBF-B associated weakly with CBF-A/CFB-C and formed a complex that was dissociated during electrophoresis. Previous characterization of cellular CBF protein present in rat liver nuclear extracts showed that CBF-B was separated easily from CBF-A and CBF-C by ion-exchange chromatography, whereas CBF-A and CBF-C were copurified after several chromatographic steps. All subunits were copurified, however, with DNA affinity chromatography (7). These findings indicated that the native CBF-A and CBF-C subunits formed a tight complex that was loosely associated with the native CBF-B subunit. Thus, the properties of subunit interactions of the recombinant CBF subunits were consistent with those of the native cellular CBF subunits.

To study association between CBF-B and CBF-A/CFB-C without separating the complex, we used the DNA-binding domain mutant CBF-B, which was previously shown to inhibit DNA binding of wild-type CBF subunits (14). Our results showed that the mutant CBF-B inhibited DNA binding of wild-type CBF subunits only in the presence of an excess quantity of nonspecific DNA. This result is consistent with the observation that mutant CBF-B, wild-type CBF-A/CFB-C, and nonspecific DNA together formed a complex. Like the mutant CBF-B, the wild-type CBF-B also formed a complex with CBF-A/CFB-C and nonspecific DNA. The amount of nonspecific DNA required to form this complex is an about 1000-fold excess over nonspecific DNA, which indicates that this is a low-affinity DNA-protein complex. Our observations indicated that CBF-B and CBF-A/CFB-C formed an unstable heterotrimeric CBF complex that was stabilized upon interaction with DNA. The association of CBF-A/CFB-C with either wild-type or mutant CBF-B was stabilized upon interaction with nonspecific DNA that formed a low-affinity DNA-protein complex. In contrast, the association

![Fig. 11. Pathway of DNA binding of the CBF subunits. CBF-A and CBF-C together form a stable CBF-A/CBF-C heterodimer, which interacts weakly with CBF-B to form an unstable heterotrimeric complex. Interaction of specific DNA with the unstable heterotrimer stabilized the association between CBF-B and CBF-A/CBF-C and formed a high-affinity DNA-protein complex. A high concentration of nonspecific DNA also stabilized the heterotrimer containing either wild-type or mutant CBF-B, indicating that the stabilization of the heterotrimer by DNA does not involve specific DNA-protein interactions.](image-url)
of CBF-A/CFB-C with the wild-type but not the mutant CBF-B was stabilized upon interaction with specific DNA that formed a high-affinity CBF-DNA complex. Under equilibrium DNA-binding conditions, therefore, CBF-A and CBF-C together formed a stable heterodimer that interacted with CBF-B to form a unstable CBF heterotrimer, which upon interaction with DNA, formed a stable DNA-protein complex (Fig. 11).

Here it is relevant to note that the subunit interaction domain of CBF-B, which consists of 21 amino acids, interacts with the CBF-A/CFB-C heterodimer but not individually with CBF-A or CBF-C. Our previous mutational analysis indicated that a hybrid surface that is generated by interaction with CBF-A and CBF-C interacts with CBF-B. The interaction between CBF-B and CBF-A/CFB-C also seemed to result in generation of a surface that interacted with DNA. This suggested that CBF subunits undergo folding transitions during the subunit interaction step. Studies of several DNA-binding proteins showed that the proteins change conformation upon specific interaction with DNA and that the structural changes are likely to be driven by binding free energy (17). Hence, it is reasonable to believe that interaction of the unstable heterotrimeric CBF with DNA facilitates the folding transitions of CBF subunits that drive the forward reactions of the DNA binding pathway.

Acknowledgment—We thank Lore Feldman for editing the manuscript.

REFERENCES
1. Dorn, A., Bollekens, J., Staub, A., Benoist, C., and Mathis, D. (1987) Cell 50, 863–872
2. Chodosh, L. A., Baldwin, A. S., Carteth, R. W., and Sharp, P. A. (1988) Cell 53, 11–24
3. Hatamoto, A., Golumbek, P. T., Van Schaftingen, E., and de Crombrugghe, B. (1988) J. Biol. Chem. 263, 5940–5947
4. Bucher, P. (1990) J. Mol. Biol. 212, 563–578
5. Maity, S. N., Golumbeck, P. T., Karsenty, G., and de Crombrugghe, B. (1988) Science 241, 582–585
6. Sinha, S., Maity, S. N., Lu, J., and de Crombrugghe, B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1624–1628
7. Maity, S. N., and de Crombrugghe, B. (1996) Methods Enzymol. 273, 217–232
8. Maity, S. N., and de Crombrugghe, B. (1998) Trends Biochem. Sci. 23, 174–178
9. Sinha, S., Kim, I.-S., Sohn, K.Y., de Crombrugghe, B., and Maity, S. N. (1996) Mol. Cell. Biol. 16, 328–337
10. Kim, I.-S., Sinha, S., de Crombrugghe, B., and Maity, S. N. (1996) Mol. Cell. Biol. 16, 4003–4013
11. Maity, S. N., and de Crombrugghe, B. (1992) J. Biol. Chem. 267, 8286–8292
12. Bi, W., Wu, L., Couny, F., de Crombrugghe, B., and Maity, S. N. (1997) J. Biol. Chem. 272, 20562–20572
13. Martinson, H. G., and True, R. J. (1979) Biochemistry 18, 1089–1094
14. Mantovani, R., Li, X.-Y., Pessara, U., van Huijsduijnen, R. H., Benoist, C., and Mathis, D. (1994) J. Biol. Chem. 269, 20540–20546
15. Arents, G., and Moudrianakis, E. N. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11170–11174
16. Luger, K., Mader, A. W., Richmond, R., Sargent, D. F., and Richmond, T. J. (1997) Nature 390, 251–260
17. Record, M. T., Jr., and Spolar, R. S. (1994) Science 263, 777–784