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The B subunit of a rat heteromeric CCAAT-binding transcription factor shows a striking sequence identity with the yeast Hap2 transcription factor

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ABSTRACT **CBF is a heteromeric mammalian transcription factor that binds to CCAAT sequences in a number of promoters such as the two type I collagen promoters, the albumin promoter, the major histocompatibility complex class II promoter, and others. It is composed of two components, A and B, that are both needed for DNA binding. We have isolated a rat cDNA containing the complete 341-amino acid coding sequence of the B component of CBF. Expression of this cDNA *in vitro* generates a polypeptide that shows the same dependency on the A component as the native B component in the formation of a complex with a CCAAT-containing DNA. The C-terminal portion of the B component from residue 260 to residue 312 shows a 75% sequence identity with a portion of the Hap2 protein, a component of a heteromeric CCAAT-binding protein in yeast. In contrast, the rest of the protein shows little sequence homology with Hap2, although both proteins contain glutamine-rich domains. In the B component of CBF this domain spans the amino-terminal 60% of the protein, whereas in Hap2 this domain is much smaller. Hence, only a few changes in one domain of this protein were tolerated during evolution between yeast and mammals, whereas the rest of the protein diverged much more extensively.**

Transcriptional control of specific genes plays a critical role in determining both normal and abnormal cellular phenotypes as well as the cellular responses to various external stimuli (1). Among the transcription factors that mediate these events are DNA-binding proteins that recognize specific sequences in gene regulatory elements (2–4). One such DNA-binding factor is the heteromeric CCAAT-binding protein (CBF) that binds to CCAAT-containing sequences that are found between −80 and −120 base pairs (bp) upstream of the start of transcription in a number of eukaryotic promoters such as the two type I collagen promoters and also the albumin, major histocompatibility complex class II, and other promoters (5–9). Since other DNA-binding proteins such as CTF/NF1 and C/EBP also recognize sequences containing CCAAT motifs, it is likely that neighboring sequences outside this CCAAT motif help discriminate between these different proteins (10, 11). CBF consists of two components, A and B (CBF-A and CBF-B), which are both needed for complex formation with DNA. CBF is a transcriptional activator and was shown, after purification by sequence-specific DNA affinity chromatography, to stimulate transcription of both the α1(I) and the α2(I) collagen promoters and other promoters in a reconstituted *in vitro* transcription system (12). It is very likely that CBF participates in the coordinate control of the two type I collagen genes. To better study the role of CBF and its mechanism of transcriptional activation, we purified the two components of CBF to apparent homogeneity with the goal of obtaining cDNAs for these subunits. We report here the characterization and sequence* of a cDNA clone for CBF-B.

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MATERIALS AND METHODS

Purification of CBF-B. Liver nuclear extracts (5) from 120 Sprague–Dawley rats were chromatographed through a 5-ml DNA affinity column containing the sequence of the mouse α2(I) collagen promoter from position −105 to position −64 linked to Sepharose. Nuclear extracts were loaded onto the affinity resin in buffer a [25 mM Hepes, pH 7.9/10% (vol/vol) glycerol/5 mM EDTA/0.5 mM phenylmethyl sulfonyl fluoride/0.5 mM dithiothreitol (DTT)/leupeptin (2 μg/ml)/pepstatin (2 μg/ml)/0.5% Nonidet P-40] plus 70 mM NaCl, and after washes with buffer a/70 mM NaCl and buffer a/350 mM NaCl, CBF activity, measured by a gel-mobility assay, was eluted with buffer a/1 M NaCl. Active fractions were diluted to 70 mM NaCl in buffer a, mixed with poly(dI-dC) (final concentration, 5 μg/ml), passed through a second affinity column containing the same DNA sequence, washed, and eluted with the same buffers as used for the first affinity chromatography. Active fractions were diluted to 70 mM NaCl in buffer b (same as buffer a except for 1 mM EDTA and 0.1% Nonidet P-40) and loaded on a 1-ml Mono S column. After washes with buffer b/100 mM NaCl and buffer b/290 mM NaCl, CBF-B was eluted by a 290 mM to 650 mM NaCl gradient. The peak CBF-B activity was eluted at 400 mM NaCl (fractions 5 and 6 of Fig. 1*A*). Fractions were assayed by the gel-mobility assay using the same α2(I) collagen double-stranded oligonucleotide that was used for the DNA affinity column. The fractions were complemented with a CBF-A preparation obtained by Mono Q chromatography of rat CBF purified once by affinity chromatography. Final purification of CBF-B was 65,000-fold with respect to total protein in liver nuclear extracts.

Southwestern Blot. (A Southwestern blot is a protein blot probed with an oligonucleotide.) Fractions were electrophoresed on a 10% polyacrylamide gel containing SDS and transferred to nitrocellulose filters. After transfer the filter was blocked with a blocking buffer [5% (wt/vol) nonfat milk/10 mM Hepes, pH 7.9/70 mM NaCl/1 mM EDTA/1 mM DTT] and incubated in binding buffer (10 mM Hepes, pH 7.9/1 mM EDTA/1 mM DTT/70 mM NaCl/0.25% nonfat milk) with a nick-translated ^{32}P-labeled catenated oligonucleotide containing the CBF binding site of the mouse α2(I) collagen promoter with and without a semi-purified CBF-A fraction.

Amino Acid Sequencing. Mono S peak CBF-B fraction (fraction 5 of Fig. 1*A*) was digested with trypsin and the resulting peptides separated by HPLC were sequenced by automatic Edman degradation. The amino acid sequence of

Abbreviations: CBF, CCAAT-binding transcription factor; CBF-A and CBF-B, A and B components of CBF, respectively; DTT, dithiothreitol; PCR, polymerase chain reaction.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34238).

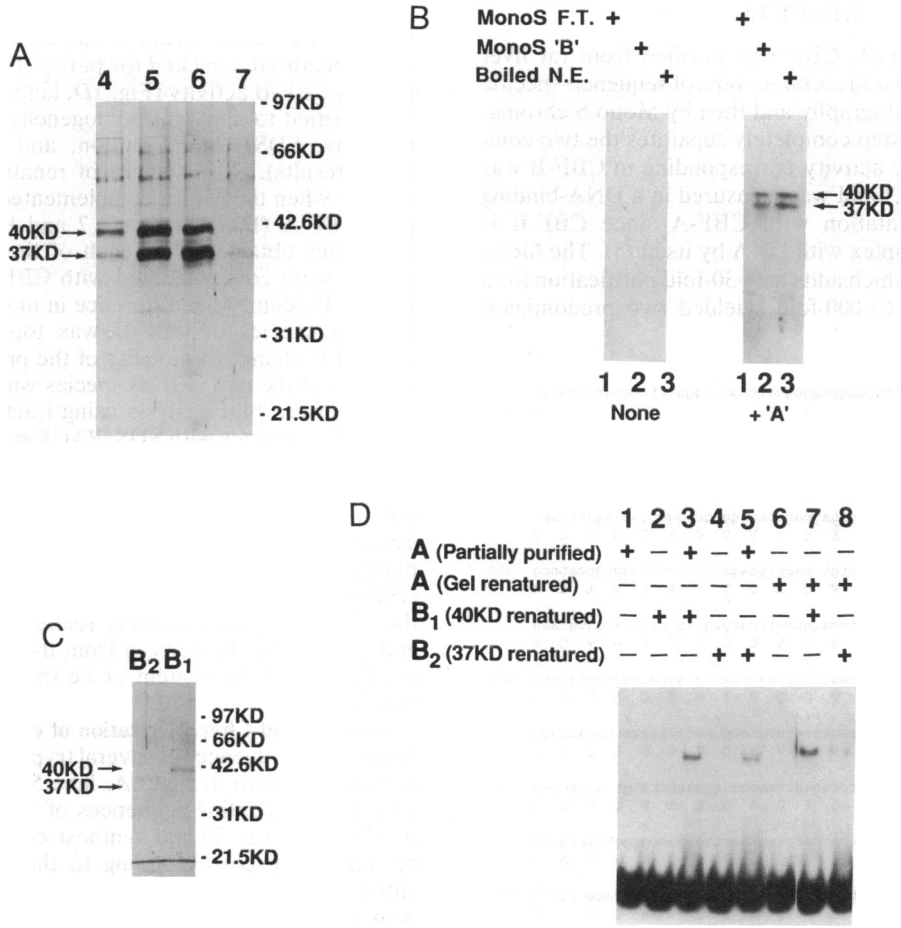

FIG. 1. Purification of CBF-B. (*A*) Chromatography of CBF-B on a Mono S column. Fractions 4–7 of the column with CBF-B activity were analyzed by SDS/PAGE. Fractions 5 and 6 contained the highest CBF-B activity. Fractions were assayed by the gel-mobility assay using a double-stranded oligonucleotide containing the mouse α2(I) collagen promoter sequence between positions −105 and −64. To determine CBF-B activity, the fractions were complemented with a CBF-A preparation. (*B*) Identification of CBF-B by Southwestern blot analysis. Two identical sets of CBF-containing fractions were electrophoresed on a 10% polyacrylamide gel containing SDS. After blotting, the filters were incubated with a nick-translated catenated oligonucleotide containing the CBF binding site of the mouse α2(I) collagen promoter, without (None) or with (+ 'A') a semi-purified CBF-A fraction. Lanes: 1, Mono S column flow-through (MonoS F.T.) after one cycle of DNA affinity chromatography containing CBF-A activity but no CBF-B activity; 2, same Mono S column high-salt wash containing CBF-B activity (MonoS 'B'); 3, boiled nuclear extract (boiled N.E.) containing CBF-B activity but no CBF-A activity. (*C*) Purification by SDS/PAGE of the two CBF-B components. Fractions 5 and 6 of Mono S column (Fig. 1*A*) were pooled and fractionated on a 10% polyacrylamide gel containing SDS. Two bands were visualized with ice-cold 0.25 M KCl/1 mM DTT, excised, and eluted separately in 0.1% SDS/50 mM Tris·HCl, pH 7.9/0.1 mM EDTA/5 mM DTT/0.2 M NaCl. Proteins were precipitated by 80% (vol/vol) acetone, washed, dried, and resuspended in 2× buffer b. A portion of each fraction was assayed for DNA-binding activity (see *D*) whereas another was analyzed again by SDS/PAGE on a 10% polyacrylamide gel. (*D*) The two renatured polypeptides of 37 kDa and 40 kDa have CBF-B activity. SDS/PAGE-purified and renatured CBF-B subunits (B_2 and B_1) were assayed by a DNA-binding gel-retardation assay in the absence and presence of CBF-A. CBF-A was either a fraction eluted from a Mono Q column after one cycle of DNA affinity chromatography (A, partially purified; lanes 1, 3, and 5) or a homogeneously pure CBF-A obtained after elution from the polyacrylamide gel and renaturation (A, gel renatured; lanes 6, 7, and 8). KD, kDa.

five tryptic peptides of various lengths was determined. Several peptides had overlapping sequences. Two peptides (Pep1 and Pep2, see Fig. 2) were chosen for oligonucleotide synthesis.

cDNA Cloning. The sense and the complementary antisense oligonucleotides, each corresponding to a portion of Pep1 and Pep2 were synthesized. The sequences of these oligonucleotides were determined with the help of a codon usage table and also by the choice of either inosine residues or degenerate bases at certain places (13, 14). The sequences of oligo1-sense and oligo1-antisense were derived from Pep1; the sequences of oligo2-sense and oligo2-antisense were derived from Pep2. Oligo1-sense, 5′-CCTGGAGCTGAGATG(C/T)TIGAGGAGGAGCC(I/C)(C/T)T(I/C)TATGT(I/C)AATGC; oligo1-antisense, 5′-GCATTCACATATA(A/G)GGGCTCCTCCTCTA(A/G)CATCTCAGCGCCAGG-3′; oligo2-sense, 5′-CACATGCAGGACCC(I/C)AACCAGGCIGATGAGGAGGC(I/C)ATGAC-3′; oligo2-antisense, 5′-GTCATGGCCTCCTCATCAGCCTGGTTGGGGTCCTGCATGTG-3′. Poly(A)$^+$-selected RNA was purified from rat liver and a first-strand cDNA was synthesized by reverse transcriptase using both oligo(dT) and random priming. cDNA (25 ng) was used in the polymerase chain reaction (PCR) using two combinations of the oligonucleotides (oligo1-sense/oligo2-antisense or oligo2-sense/oligo1-antisense). Major DNA bands obtained after 40 cycles of amplification were cloned by blunt-end ligation and sequenced. One of these PCR clones, obtained with the oligo1-sense/oligo2-antisense combination, contained sequences adjacent to the primer sequences that encoded the expected amino acid sequences in Pep1 and Pep2. This PCR clone of 260 bp was used to obtain a 2.5-kilobase (kb) cDNA by screening 1×10^6 plaques of a rat liver cDNA library constructed in λZAP (Stratagene). After purification, the vector/insert was rescued from λ arms to the phagemid state (Stratagene) (15). The PCR clone contained the sequence between residue 915 and residue 1174 (Fig. 2).

RESULTS

Purification of CBF-B. CBF was purified from rat liver nuclear extracts by two successive steps of sequence-specific DNA affinity chromatography and then by Mono S chromatography. The latter step completely separates the two components of CBF. The activity corresponding to CBF-B was eluted at 0.35 M NaCl and was measured in a DNA-binding assay by complementation with CBF-A since CBF-B is unable to form a complex with DNA by itself (5). The Mono S chromatography, which adds an ≈30-fold purification for a total purification of 65,000-fold, yielded two predominant protein species of 40 and 37 kDa (Fig. 1*A*). Each of these two proteins was eluted from a polyacrylamide gel containing SDS, renatured, checked for purity (Fig. 1*C*), and shown to possess CBF-B activity (Fig. 1*D*, lanes 3 and 5). CBF-A was also purified to apparent homogeneity by Mono Q chromatography, SDS/PAGE, elution, and renaturation (unpublished results). Both species of renatured CBF-B regained activity when they were complemented with this apparently pure CBF-A (Fig. 1*D*, lanes 7 and 8). The protein–DNA complexes obtained after each of the two purified CBF-B species were complemented with CBF-A had identical mobilities. Evidently the difference in molecular mass between the two species of CBF-B was too small to produce a detectable change in mobility of the protein–DNA complex. The size of the two CBF-B species was also confirmed by a Southwestern blot analysis using fractions with CBF-B activity. Two species with SDS/PAGE mobilities of 40 kDa and 37 kDa bound to specific double-stranded DNA oligonucleotides containing the α2(I) collagen promoter CCAAT motif only when CBF-A was present in the DNA-binding solution. These same two species were detected both with highly purified CBF-B (Fig. 1*B*, lane 2, + 'A') and with a boiled crude nuclear extract (Fig. 1*B*, lane 3, + 'A'). We believe that the 37-kDa species is closely related to the 40-kDa species and could either be derived from the 40-kDa species during purification of the protein or be the product of alternative splicing.

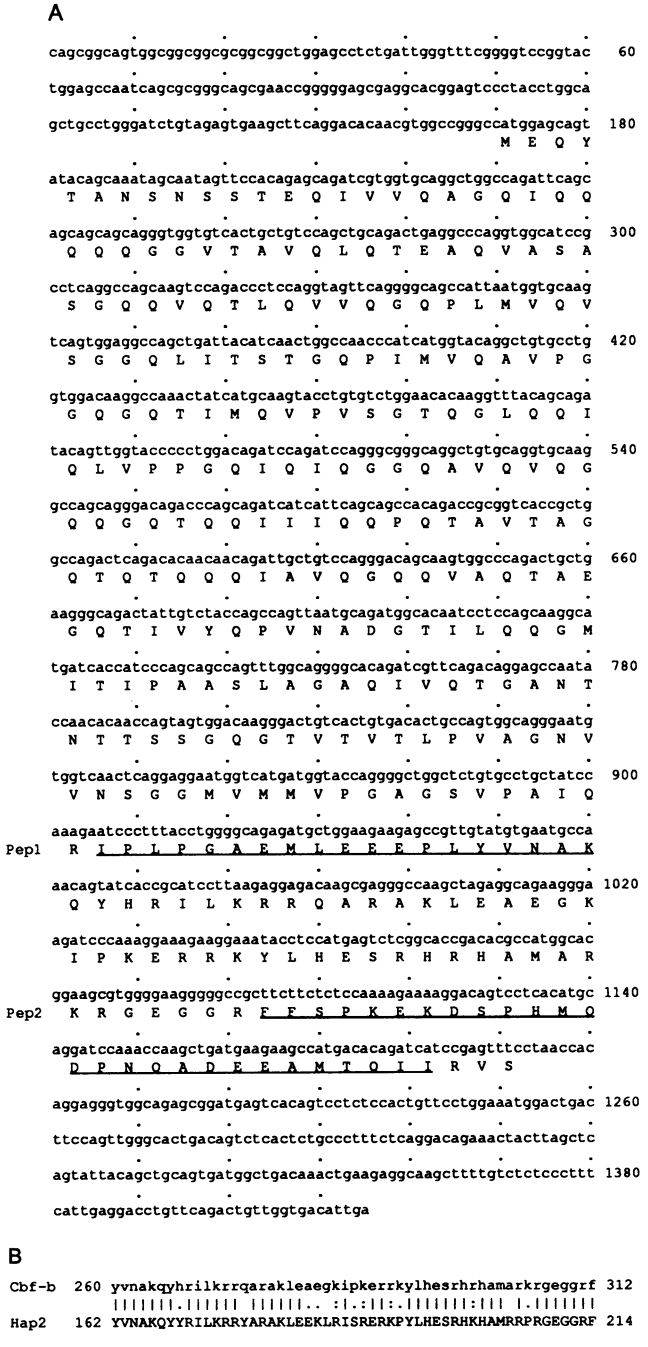

FIG. 2. (*A*) DNA sequence of cDNA clone pCBF-B1. The figure shows the sequence of the 5′ proximal 1415 bp of this cDNA. The entire 2.5-kb sequence of pCBF-B1 has been deposited in the GenBank data bank. The amino acid sequences (single-letter code) of the two tryptic peptides Pep1 and Pep2 are underlined. (*B*) Comparison of the amino acid sequences of rat CBF-B between residues 260 and 312 and of yeast Hap2 between residues 162 and 214.

Isolation and Characterization of cDNA Clones for CBF-B. Amino acid sequences of several tryptic peptides of the Mono S fraction shown in Fig. 1*A*, lane 5, were determined. We selected two peptide sequences of 19 (Pep1) and 27 (Pep2) residues (see Fig. 2) and synthesized oligonucleotides of 44 bp and 41 bp corresponding to the sense strand and the antisense strand, respectively. These oligonucleotides were then used pairwise in PCR experiments in which the initial template was a cDNA preparation of rat liver poly(A)$^+$ mRNA. In one PCR cDNA species of 260 bp, the cDNA sequences adjacent to the oligonucleotide primers coded for the expected amino acid sequences present in the two tryptic peptides. In addition, the deduced amino acid sequence of this 260-bp cDNA presented a high degree of sequence identity with a portion of the yeast transcription factor Hap2 (16). The yeast Hap2 polypeptide is a component of a heteromeric CCAAT-binding protein (17). This 260-bp PCR cDNA was used as hybridization probe to obtain a larger cDNA of 2.5 kb. The sequence of part of this cDNA is shown in Fig. 2 and contains an open reading frame of 1023 bp encoding a protein of 341 amino acids starting with a methionine at nucleotide 171 of the cDNA. The calculated molecular mass of the deduced polypeptide is 41 kDa, in excellent agreement with the molecular mass of the largest of the two CBF-B polypeptides measured by SDS/PAGE.

To test whether a functional CBF-B could be synthesized *in vitro* using the cloned cDNA, we transcribed the 2.5-kb cDNA with bacteriophage T3 RNA polymerase and translated the RNA in a reticulocyte lysate. By SDS/PAGE, the size of the [^{35}S]methionine-labeled translation product was very similar to the size of the 40-kDa native CBF-B (Fig. 3*B*). We first determined that the reticulocyte lysate contained neither CBF activity (Fig. 3*A*, lane 4) nor CBF-B activity (Fig. 3*A*, lane 5) and only traces of CBF-A activity (Fig. 3*A*, lane 6), which were only detectable after long exposures of the autoradiograph. Furthermore, the reticulocyte lysate did not inhibit CBF activity (compare lanes 3 and 7). When assayed for DNA binding, the translated CBF-B showed no DNA-binding activity by itself (Fig. 3*A*, lanes 8 and 9) but when complemented with CBF-A, DNA-binding activity was demonstrated (Fig. 3*A*, lanes 10 and 11). This activity increased with increasing amounts of translated CBF-B and was seen when CBF-A was present during the translation of

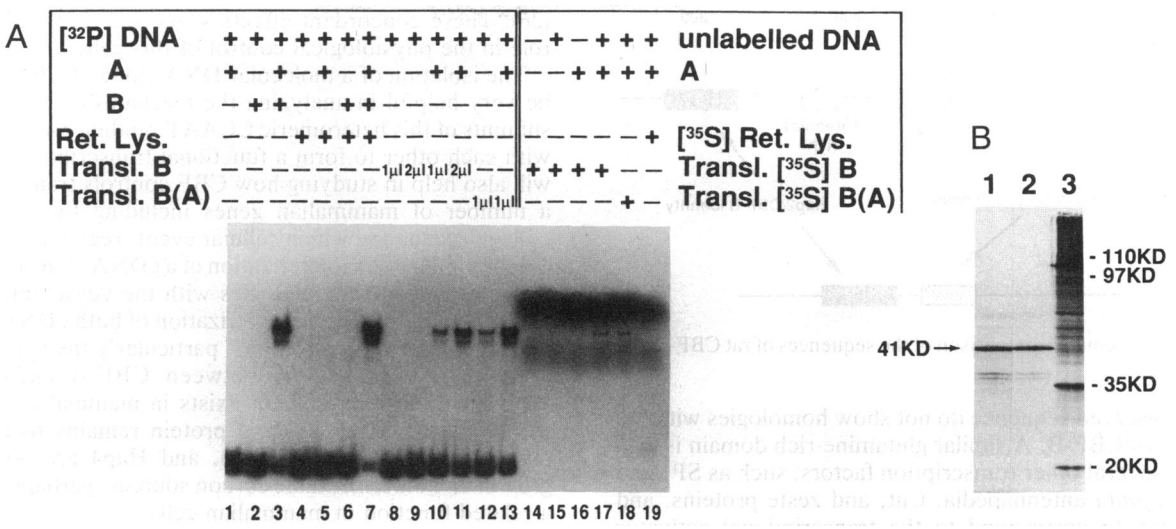

FIG. 3. (*A*) The cDNA of pCBF-B1 specifies a functionally active CBF-B. DNA-binding assay of the products of transcription–translation. The 2.5-kb cDNA clone, pCBF-B1, was linearized by *Xho* I and transcribed by T3 RNA polymerase (Stratagene) in presence of the capping analog 7-methylguanosine(5′)triphospho(5′)-2′-*O*-methylguanosine. The resulting RNA was translated in a rabbit reticulocyte lysate (Promega) in the presence (lanes 12 and 13) or absence (lanes 8–11) of purified CBF-A. The translated product in the reticulocyte lysate was assayed for CBF-B activity by complementation with CBF-A (lanes 10, 11, and 13). The translated product labeled by [^{35}S]methionine in a separate reaction was also assayed for CBF-B activity (lanes 14–19). In these lanes, the smears above and below the band corresponding to the protein–DNA complex are due to translation products that are RNA template-independent (lane 19). A, Mono Q fraction after DNA affinity chromatography; B, Mono S fraction (see Fig. 1*A*); Ret. Lys., reticulocyte lysate; Transl. B, products of transcription/translation of pCBF-B1; Transl. B (A), same as Transl. B. except fraction A was added to the reticulocyte lysate before the translation reaction. (*B*) SDS/PAGE of *in vitro*-translated ^{35}S-labeled proteins after transcription/translation of cDNA clone pCBF-B1. ^{35}S-labeled translated products were fractionated in a 10% polyacrylamide gel containing SDS. Lanes: 1, translation products of CBF-B RNA; 2, translation products of CBF-B RNA translated in presence of CBF-A protein; 3, translation products of brome mosaic virus RNA. No discrete ^{35}S-labeled proteins were seen in translation reactions without RNA. KD, kDa.

CBF-B and when CBF-A was added after its synthesis (Fig. 3*A*, lanes 12 and 13). When native CBF-A and CBF-B were added to form a complex with the CCAAT-containing test oligonucleotide, the protein–DNA complex appeared as two bands of unequal intensity (Fig. 3*A*, lane 3). Complexes of identical mobilities were observed when translated CBF-B was used instead of native CBF-B. These experiments indicate that the 2.5-kb cDNA clone encodes a polypeptide with the same molecular mass as purified native CBF-B, and that this *in vitro*-synthesized polypeptide is capable of generating the same complex with CBF-A and DNA and displays the same dependency on CBF-A for DNA–protein complex formation.

To demonstrate that CBF-B was present in the DNA–protein complex the ^{35}S-labeled translated CBF-B was added with native CBF-A to an unlabeled CCAAT-containing test oligonucleotide. After gel retardation analysis, ^{35}S-labeled CBF-B was found at exactly the same position in the gel as the [^{32}P]DNA–CBF complex in a conventional gel-retardation assay (Fig. 3*A*, lanes 17 and 18). As predicted, this occurred only when all three components, translated CBF-B, native CBF-A, and test DNA, were present in the DNA-binding reaction. This specific mobility was not shown after gel electrophoresis by translated ^{35}S-labeled CBF-B (lane 14), translated ^{35}S-labeled CBF-B added to test DNA (lane 15), or translated CBF-B added to CBF-A (lane 16).

Northern hybridization of rat liver RNA showed a single discrete species of CBF-B RNA at ≈3.8 kb (Fig. 4), clearly larger than the ≈1-kb sequence needed to code for CBF-B. We also screened a limited number of additional rat tissues by Northern blot hybridization, including thymus, testis, and mouse embryos. In each of these samples, the 3.8-kb RNA species was present although its concentration appeared to vary in the various samples (data not shown). The same heteromeric CCAAT-binding protein was present in fibroblasts in culture (5).

Homology with the Yeast Hap2 Transcription Factor. The amino acid sequence of the 341-residue CBF-B polypeptide shows several interesting features. The sequence of the C-terminal end of the protein between residues 260 and 312 shows a 75% sequence identity with a segment of the yeast Hap2 protein (16) between residues 162 and 214 (Figs. 2*B* and 5). The segment of sequence identity with Hap2 is very hydrophilic and rich in basic residues, consistent with the possibility that it contains a DNA-binding domain. Not surprisingly, the predicted secondary structure of this segment (based on the PeptideStructure program) was also very similar to that of the conserved segment in Hap2, displaying two potential α helices interrupted by a very short sequence. The segment of CBF-B from residue 14 to residue 161 is very rich in glutamine (51 out of 146 residues or 35%). In Hap2 a much smaller segment between residues 119 and 148 is also rich in glutamines including a stretch of 14 glutamine residues in a row (see Fig. 5). However, the overall sequence of this segment and that of other segments of Hap2 outside the

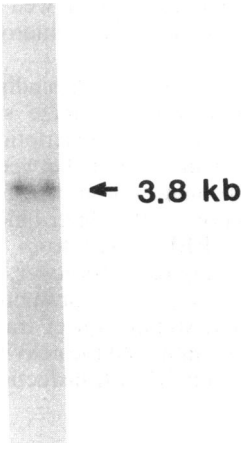

FIG. 4. Northern blot hybridization of CBF-B RNA. Rat liver poly(A)$^+$ RNA (15 µg) was fractionated on a 0.75% agarose/formaldehyde gel (18). After transfer to a GeneScreen*Plus* membrane the RNAs were hybridized with nick-translated ^{32}P-labeled CBF-B$_1$ DNA. RNA markers in a parallel lane were used to calculate the size of CBF-B RNA.

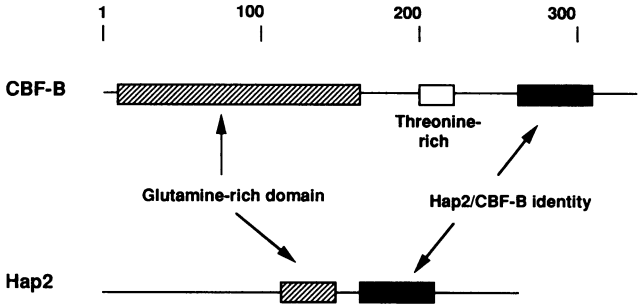

FIG. 5. Schematic comparison of the sequences of rat CBF-B and yeast Hap2.

highly conserved sequence do not show homologies with the sequence of CBF-B. A similar glutamine-rich domain is also found in several other transcription factors, such as SP1 and the *Drosophila* antennapedia, Cut, and zeste proteins, and was shown to correspond to the transcriptional activator domain of these proteins (19–22). This could suggest, by analogy, that the glutamine-rich segment of CBF-B also specifies a transcription activation domain. On the C-terminal side of the glutamine-rich segment in CBF-B is a small threonine-rich segment (residues 200–216). Two threonine-rich segments are also found in SP1 adjacent to the glutamine-rich segments (19). We noted that CBF-B does not show regularly spaced leucine residues, as seen in leucine zipper proteins (23). Furthermore, there is no sequence homology between CBF-B and two other CCAAT-binding proteins C/EBP or CTF/NF1 (10, 11).

DISCUSSION

CBF-B protein thus contains a domain that is extraordinarily well conserved between yeast and mammals. This domain must specify one or more functions that were also highly conserved between yeast and mammals. Hap2 is part of a multisubunit CCAAT-binding transcription factor in yeast that in this species contains at least three components (Hap2, Hap3, and Hap4), all of which are present in the DNA–protein complex (17, 24). Extracts containing Hap2 prepared from Hap3-minus yeast cells can complement a chromatographic fraction of HeLa cell extracts containing CP1-B, the likely human homologue of CBF-A, to form a complex with a CCAAT sequence (25). It is, therefore, highly probable that the conserved domain in CBF-B contains an interaction site for CBF-A and possibly also a DNA-binding domain. Interestingly, the overall sequence and the size of the glutamine-rich domain showed considerable divergence between the two species. Therefore, in one segment of this transcription factor, which contains most probably a subunit-interaction domain and possibly a DNA-binding domain, only very few changes were tolerated during evolution between yeast and mammals. In contrast, many more changes were allowed in the rest of the protein including the glutamine-rich putative transcriptional activation domain.

In addition to CBF, at least two other DNA-binding proteins, C/EBP and CTF/NF1 (10, 11), recognize sequences that contain a CCAAT motif, but these transcription factors appear at least in some cases to differentiate between the various binding sites. In the α2(I) collagen promoter, for instance, the CCAAT motif at position −80, which binds CBF, did not bind CTF/NF1 or C/EBP (a gift from S. McKnight, Carnegie Institution, Baltimore). Moreover, a mutation in this CCAAT motif of the α2(I) collagen promoter that abolished CBF binding prevented stimulation of transcription by CBF (12), and also strongly inhibited the activity of the promoter in intact fibroblasts after DNA transfection (26). These concordant effects suggested that CBF plays a role in the physiological control of this gene.

The isolation of a molecular DNA clone of CBF-B should be very helpful in analyzing the mechanisms by which the subunits of this heteromeric CCAAT-binding protein interact with each other to form a functional transcription factor. It will also help in studying how CBF controls transcription of a number of mammalian genes including the two type I collagen genes and which cellular events regulate its activity. The preliminary characterization of a cDNA clone for CBF-A indicates sequence homologies with the yeast transcription factor Hap3. Further characterization of both cDNAs should help elucidate these questions, particularly the nature of the heteromeric interactions between CBF-A and CBF-B. Whether a third component exists in mammalian CBF homologous to the yeast Hap4 protein remains to be determined. In yeast, Hap2, Hap3, and Hap4 are needed for growth on nonfermentable carbon sources; perhaps CBF has a related function in mammalian cells.

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