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A Cloned Human CCAAT-Box-Binding Factor Stimulates Transcription from the Human hsp70 Promoter

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The basal promoter of the human hsp70 gene is predominantly controlled by a CCAAT element at position −70 relative to the transcriptional initiation site. We report the isolation of a novel cDNA clone encoding a 114-kDa polypeptide that binds to the CCAAT element of the hsp70 promoter. Expression of this CCAAT-binding factor (CBF) cDNA activated transcription from cotransfected hsp70 promoter-reporter gene constructs in a CCAAT-dependent manner. CCAAT-binding factor shows no homology to the previously identified human CCAAT transcription factor or rat CCAAT/enhancer-binding protein.

Expression of the human hsp70 gene is cell cycle regulated (24) and is induced by both serum and the adenovirus E1a protein (37, 39). This regulated expression is conferred by the basal promoter, whose activity is predominantly controlled by the CCAAT element at position −70 relative to the transcription initiation site (14, 35, 38). The CCAAT homology was originally identified as a potential *cis*-acting promoter element for a number of eucaryotic genes (2, 9), and since then multiple CCAAT-binding activities have been reported (1, 6, 7, 29). Although it is difficult to assess the relationship among these activities, the data suggest that different CCAAT elements are recognized in vivo by different factors.

The best-characterized CCAAT-binding proteins are CCAAT transcription factor/nuclear factor 1 (CTF/NF1) and CCAAT/enhancer-binding protein (C/EBP). CTF/NF1 is a set of HeLa cell nuclear polypeptides ranging from 52 to 66 kDa that function both as transcription factors for in vitro transcription of α-globin promoter templates and as in vitro initiation factors for adenovirus DNA replication (18). C/EBP is a heat-stable, 42-kDa (42K) rat liver nuclear protein that shows selective binding to the CCAAT homology of several viral promoters as well as to the core homology common to many viral enhancers (13, 17). These two proteins are not related, as judged by their predicted amino acid sequences derived from cDNA clones (20, 30), and appear to use different structural features to facilitate DNA-binding, C/EBP by the dimerization leucine zipper motif (21) and CTF/NF1 by as yet an undefined structure.

To obtain cDNA clones encoding factors that specifically bind to the hsp70 CCAAT element, we screened a λgt11 cDNA expression library with an oligonucleotide corresponding to the CCAAT element of the hsp70 promoter (31, 34). We report the isolation of a cDNA clone encoding a 999-amino-acid polypeptide corresponding to a molecular mass of 114 kDa. When expressed in bacteria, this polypeptide binds to the hsp70 promoter in a CCAAT-sequence-specific manner. When expressed in mammalian cells, this polypeptide stimulates transcription from the hsp70 promoter in a CCAAT-element-dependent manner. The predicted amino acid sequence of this CCAAT-binding factor (CBF) is distinct from those of the two previously cloned CCAAT-binding proteins, CTF/NF1 (30) and C/EBP (20).

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MATERIALS AND METHODS

Bacterial expression. pGEX-N1/534 and pGEX-CBF were constructed in plasmid pGEX2T (33). pGEX-N1/534 encodes the fusion protein glutathion-S-transferase (GST)-N1/534, containing residues 1 to 534 of CBF, and was used to generate a rabbit polyclonal antiserum. pGEX-CBF encoded fusion protein GST-CBF, containing residues 1 to 999 of CBF, and was used for in vitro binding assays.

Induction and purification of the fusion proteins were accomplished by a procedure modified from that of Smith and Johnson (33). Cells were induced with 3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C before being harvested by centrifugation. The pellet from a 300-ml culture was resuspended in 8 ml of 8.5% sucrose–2 mM $MgCl_2$–0.25 M Tris (pH 8.0) and lysed with 32 mg of lysozyme. Incubation on ice with 3.2 ml of 0.25 M EDTA for 30 min was done prior to the addition of 9 ml of 0.5% Triton X-100–62.5 mM EDTA–50 mM Tris (pH 8.0). Phenylmethylsulfonyl fluoride, dithiothreitol, and ethyleneglycoltetraacetic acid were added to final concentrations of 1, 10, and 20 mM, respectively. The cell lysate was cleared by centrifugation at 26,800 × *g* for 1 h at 4°C. The lysate was incubated with 800 μl of 50% glutathione agarose beads (Sigma) for 12 h at 4°C on a rotating platform. The beads were washed three times in phosphate-buffered saline before elution of the fusion protein with 2 ml of 50 mM glutathione–50 mM Tris (pH 7.5) for 30 min at 25°C. The eluted protein was concentrated, and the buffer was exchanged for phosphate-buffered saline by repeated dilutions and concentrations by Amicon filtration.

cDNA library screening. A λgt11 expression library prepared from human WI38 cells was screened with end-labeled wild-type (wt) CCAAT oligonucleotides by the procedure of Vinson et al. (34). For DNA hybridization screening (22), the 2.1-kb insert of λC53 was labeled by nick translation and used to probe a λZAP cDNA library prepared from human U2-OS cells.

DNA sequencing and computer analyses. cDNA was excised from λgt11 phage by restriction digestion and subcloned into the polylinker of pSP73 (Promega). cDNA was excised from λZAP phage according to the protocol of the

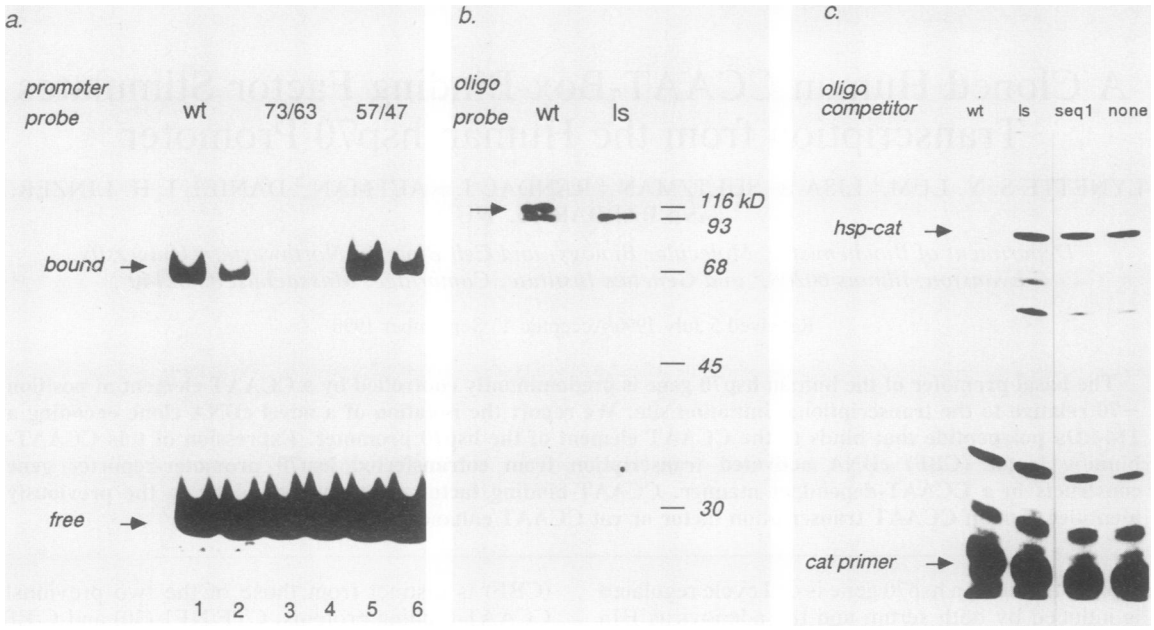

FIG. 1. Demonstration that the CCAAT element of the hsp70 promoter is a sequence-specific binding site for a transcription factor. (a) Gel mobility shift assay directed by wt (lanes 1 and 2), ls 73/63 (lanes 3 and 4), and ls 57/47 (lanes 5 and 6) promoter probes incubated with 10 μg (lanes 1, 3, and 5) or 5 μg (lanes 2, 4, and 6) of HeLa cell nuclear extracts. Promoter probes extend from the *Ssp*I site at position −104 to the *Hpa*II site at position −4 of the hsp70 promoter and are labeled at the *Hpa*II site. (b) Southwestern assays of HeLa cell nuclear extracts probed with synthetic double-stranded oligonucleotides (oligo) corresponding to the wt or mutant ls CCAAT element of the hsp70 promoter. The arrow indicates the position of sequence-specific binding activity. (c) In vitro transcription directed by the hsp70 promoter in a HeLa cell nuclear extract in the absence (none) or in the presence of a 10-fold molar excess, relative to the template, of wt or ls CCAAT oligonucleotides or a nonspecific oligonucleotide (seq1). Transcription products were analyzed by primer extension.

manufacturer (Stratagene). Further subcloning was accomplished by using convenient restriction sites. Sequencing reactions were performed by double-stranded dideoxynucleotide sequencing with an SP6, T7, or T3 primer. Sequence analyses were performed with the software package of the University of Wisconsin Genetics Computer Group. Detection of protein signatures and sites was performed with PROSITE. The consensus sequences used for potential phosphorylation sites were as follows: (K,R)-(K,R)-X-(S,T) for cyclic (cAMP)-cGMP-dependent kinase; (S,T)-X-X-(E,D) for casein kinase II; (R,K)-X2-(E,D)-X3-Y or (R,K)-X3-(E,D)-X2-Y for tyrosine kinase; and (S,T)-X-(R,K) for protein kinase C.

Gel shift and exonuclease III protection assays. Binding reactions and gel electrophoresis for the gel shift assays were conducted essentially as previously described (32), with the substitution of 0.5× TBE (1× TBE is 89 mM Tris–89 mM boric acid–2 mM EDTA) for gel electrophoresis. Promoter probes and exonuclease III protection assays were prepared as previously described (40). Equivalent amounts of bacterially produced GST and GST-CBF polypeptides were confirmed by Coomassie staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Binding reactions with 100 ng of bacterially produced GST or GST-CBF were supplemented with 8 μg of bovine serum albumin.

Metabolic labeling and immunoprecipitations. At 48 h posttransfection, COS cells were labeled with 125 μCi of ^{35}S translabel (ICN) per ml for 2 h. Cells were lysed in Laemmli sample buffer (19) and diluted 1:10 in RIPA buffer for denaturing immunoprecipitations (25).

Preparation of nuclear extract, in vitro transcription, and Southwestern (DNA-protein) analysis. HeLa cell nuclear extracts were prepared as described by Dignam et al. (5). Transcription reactions and primer extension analysis were conducted as previously described (27). The template for transcription is hsp-cat (38), containing sequences from positions −150 to +150 of the hsp70 gene fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. For Southwestern assays, HeLa cell nuclear extracts were fractionated by SDS-PAGE and transferred to nitrocellulose filters. All subsequent steps were conducted at 4°C. The filters were preincubated in binding buffer (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-KOH [pH 7.4], 5 mM $MgCl_2$, 2 mM dithiothreitol, 110 mM NaCl)–5% nonfat dry milk–5× Denhardt solution (1× Denhardt solution is 0.02% Ficoll–0.02% polyvinylpyrrolidone–0.02% bovine serum albumin) for 30 min; probed with the indicated end-labeled oligonucleotide at 100 fmol/ml in binding buffer with 0.25% nonfat dry milk, 5× Denhardt solution, 10 μg of sheared salmon sperm DNA per ml, and 10 μg of poly(dI-dC) (Pharmacia) per ml for 2 h; and washed with four changes of binding buffer for 20 min.

Oligodeoxynucleotides. Oligonucleotides used were the following:

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wt CCAAT: gatctCTCGGTGATTGGCTCAGAAGGa
                aGAGCCACTAACCGAGTCTTCCtctag
ls CCAAT: gatctCcgaGatcTcGGCTCAGAAGGa
                aGgctCtagAgCCGAGTCTTCCtctag
seq1:   gatctCAGAAGGGAAAa
                aGTCTTCCCTTTtctag
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Synthesis of cRNA, selection of mRNA, and translation of CBF in vitro. The 3,002-bp *Nco*I fragment of CBF, containing nucleotides 10 to 3012 and encoding amino acids 1 to 999, was subcloned into pT7βSal (28), which provides a β-globin leader that improves translation from a linked coding region. In vitro transcription with T7 RNA polymerase in the

presence of m7GppG was performed as described previously (23). CBF mRNA from HeLa cells was purified by hybridization to the insert from λC53. Selection of CBF mRNA was performed as previously described (36). In vitro translation using rabbit reticulocyte lysates was done according to the manufacturer's instructions.

Expression constructs and transfections. COS cell transfections (4) and CAT assays (12) were performed as described previously.

pMT2-CBF was constructed in two steps. The 2,140-bp *Xba*I fragment from λC53 was inserted into the *Xba*I site in pMT2, resulting in pMT2-CX. The 304-bp *Pst*I-to-*Hin*dIII fragment from pMT2-CX was excised and ligated with the 2,728-bp *Hin*dIII-*Xba*I fragment from λ9A into the *Pst*I and *Xba*I sites in pMT2, resulting in pMT2-CBF.

Target promoter-CAT gene constructs were as follows: wt, the wt hsp70 promoter from positions −100 to + 150; 73/63, a linker substitution in the CCAAT element at position −70 of the hsp70 promoter (40); 87, the α-globin promoter from positions −87 to +36; 55, a CCAAT-box-deleted α-globin promoter (30).

Nucleotide sequence accession number. The GenBank accession number for the sequence reported is M37197.

RESULTS

Identification of a CCAAT-binding protein. In the context of the human hsp70 promoter, the CCAAT element at position −70 is a sequence-specific binding site in vitro for HeLa cell nuclear proteins, as demonstrated by exonuclease III protection (40) or electrophoretic mobility shift (Fig. 1a) assays. The shifted species detected with a wt promoter probe was abolished when a probe containing a linker scan (ls) mutation that destroys the CCAAT element (73/63) was used. The binding specificity was due to the disruption of the CCAAT element and not to the introduction of linker sequences, since the identical sequence mutation introduced elsewhere (57/47) did not disrupt the shifted species. These observations enabled us to design synthetic double-stranded oligonucleotides corresponding to the wt CCAAT-binding site and the mutated ls CCAAT site to probe HeLa cell nuclear extracts in Southwestern assays (Fig. 1b). The wt probe bound most prominently to polypeptides of 116 and 93 kDa, although larger polypeptides were also detected. The specificity of binding is illustrated by the pattern detected with the ls probe: a 93-kDa polypeptide at an intensity equivalent to that detected with the wt probe, indicative of a nonspecific interaction, and a 116-kDa polypeptide at an intensity 60-fold less than that observed with the wt probe, indicative of a specific interaction. The relationship among the polypeptides showing specific binding is unknown. They could be distinct proteins varying in abundance or with different affinities for the probe; alternatively, they may represent proteolytic fragments or modified forms of a single protein. The wt oligonucleotide also interfered with in vitro transcription directed by the hsp70 promoter, resulting in a 15- to 20-fold reduction in accumulated transcripts (Fig. 1c), presumably by competing with the promoter for a limiting transcription factor. This competition was specific, since oligonucleotides ls and seql, a nonspecific oligonucleotide, had no effect. These observations suggest that the proteins capable of binding the wt oligonucleotide are likely to function as CCAAT-binding transcription factors.

Isolation of cDNA clones encoding a CCAAT-binding protein. A cDNA library prepared from human WI38 cells, derived from human embryonic lung tissue, was screened for

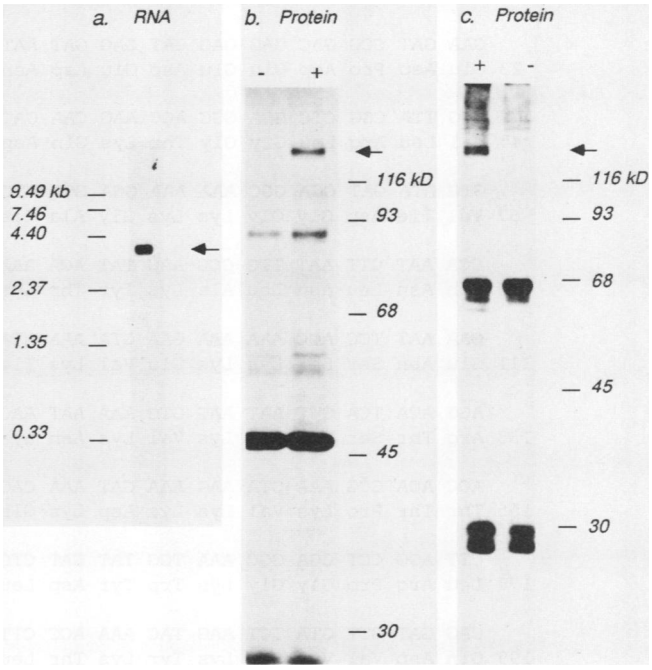

FIG. 2. (a) Northern blot analysis of total RNA prepared from HeLa cells and probed with the insert from λC53. (b) Hybrid-selected translation assay of HeLa cell poly(A)$^+$ RNA. RNA complementary to the insert from λC53 was selected by hybridization and translated in vitro in a rabbit reticulocyte lysate supplemented with [^{35}S]methionine. Translation products of endogenous (−) or hybrid-selected (+) RNA were fractionated by SDS-PAGE. (c) In vitro translation in rabbit reticulocyte lysate of T7-transcribed CBF sense (+) or antisense (−) cRNA. Arrows indicate the positions of CBF polypeptide translated from mRNA (b) and cRNA (c).

binding to the wt CCAAT oligonucleotide by the procedure of Vinson et al. (34). From approximately 500,000 plaques, two positive plaques, containing inserts of 2.1 and 1.6 kb, were isolated. Lysogens were generated from each recombinant phage, and extracts were prepared to confirm the sequence-specific DNA-binding activity in Southwestern assays (data not shown). All subsequent analyses were conducted on the larger clone, λC53. The sequence of λC53 revealed an uninterrupted 2,140-nucleotide open reading frame with a coding capacity for a 78-kDa polypeptide. However, the lack of a translational termination codon, the detection of a 3.8-kb RNA species in HeLa cells by Northern (RNA) analysis (Fig. 2a), and the hybrid-selected translation product with an electrophoretic mobility greater than 116 kDa (Fig. 2b) suggested that λC53 is only a partial cDNA. A second library, prepared from human U2-OS cells, derived from human bone osteogenic sarcoma, was therefore screened by DNA hybridization, using the λC53 insert as the probe. Four additional clones were isolated: λ3B (nucleotides 25 to 2638), λ9A (nucleotides 26 to 3132), λ1151 (nucleotides 1623 to 3244), and λ141 (nucleotides 400 to 2635). The nucleotide sequences of these clones are identical with the exception of nucleotides 2622 to 2638. In λ141 and λ3B, this region is composed of A residues. Given that this is the 3′ end of λ141 and λ3B, this poly(A) stretch may represent an alternative 3′ poly(A) addition site or may have resulted from a cloning artifact.

The composite sequence of 3,244 nucleotides derived from these overlapping clones contains a potential initiation codon at nucleotide 12, a termination codon at nucleotide

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GCTTTGCCGCC ATG GCC GCA GTC AAG GAG CCT TTG GAG TTC CAT GCC AAG CGG CCT TGG CGC CCC GAG GAG GCA GTA   77
          1 MET Ala Ala Val Lys Glu Pro Leu Glu Phe His Ala Lys Arg Pro Trp Arg Pro Glu Glu Ala Val

            GAA GAT CCG GAC GAG GAG GAT GAG GAT AAT ACT AGT GAA GCC GAG AAT GGG TTC TCC CTG GAG GAA   143
           23 Glu Asp Pro Asp Glu Glu Asp Glu Asp Asn Thr Ser Glu Ala Glu Asn Gly Phe Ser Leu Glu Glu

            GTG TTA CGG CTC GGA GGC ACC AAG CAA GAT TAC CTT ATG CTG GCT ACT TTG GAT GAG AAT GAG GAA   209
           45 Val Leu Arg Leu Gly Gly Thr Lys Gln Asp Tyr Leu MET Leu Ala Thr Leu Asp Glu Asn Glu Glu

            GTG ATA GAT GGA GGC AAA AAA GGA GCA ATC GAT GAC CTT CAG CAA GGT GAA TTG GAA GCA TTT ATT   275
           67 Val Ile Asp Gly Gly Lys Lys Gly Ala Ile Asp Asp Leu Gln Gln Gly Glu Leu Glu Ala Phe Ile

            CCA AAT CTT AAT TTG GCG AAG TAT ACA AAA GCT TCC TTA ATT GAA GAA GAT GAA CCA GCT GAA AAA   341
           89 Gln Asn Leu Asn Leu Ala Lys Tyr Thr Lys Ala Ser Leu Ile Glu Glu Asp Glu Pro Ala Glu Lys

            GAA AAT TCC AGC AAA AAA GAA GTA AAA ATA CCT AAA ATA AAT AAT AAA AAT ACA GCA GAA AGT CAA   407
          111 Glu Asn Ser Ser Lys Lys Glu Val Lys Ile Pro Lys Ile Asn Asn Lys Asn Thr Ala Glu Ser Gln

            AGG ACA TCA GTT AAT AAG GTG AAA AAT AAG AAT AGG CCA GAA CCA CAT TCT GAT GAG AAT GGC AGT   473
          133 Arg Thr Ser Val Asn Lys Val Lys Asn Lys Asn Arg Pro Glu Pro His Ser Asp Glu Asn Gly Ser

            ACC ACA CCG AAA GTA AAG AAA GAT AAA CAG AAC ATC TTT GAA TTT TTT GAG AGA CAG ACT TTG TTA   539
          155 Thr Thr Pro Lys Val Lys Lys Asp Lys Gln Asn Ile Phe Glu Phe Phe Glu Arg Gln Thr Leu Leu

            CTT AGG CCT GGA GGC AAA TGG TAT GAT CTG GAG TAC AGC AAT GAA TAT TCT TTG AAA CCC CAG CCT   605
          177 Leu Arg Pro Gly Gly Lys Trp Tyr Asp Leu Glu Tyr Ser Asn Glu Tyr Ser Leu Lys Pro Gln Pro

            CAG GAT GTT GTA TCT AAG TAC AAA ACC CTT GCT CAG AAG CTG TAT CAG CAT GAA ATC AAC TTA TTC   671
          199 Gln Asp Val Val Ser Lys Tyr Lys Thr Leu Ala Gln Lys Leu Tyr Gln His Glu Ile Asn Leu Phe

            AAA AGT AAG ACG AAT AGT CAA AAG GGA GCC TCT TCT ACC TGG ATG AAG GCA ATT GTG TCA TCG GGG   737
          221 Lys Ser Lys Thr Asn Ser Gln Lys Gly Ala Ser Ser Thr Trp MET Lys Ala Ile Val Ser Ser Gly

            ACA CTA GGT GAC AGG ATG GCA GCC ATG ATT CTT CTT ATT CAG GAT GAT GCC GTT CAC ACA CTT CAG   803
          243 Thr Leu Gly Asp Arg MET Ala Ala MET Ile Leu Leu Ile Gln Asp Asp Ala Val His Thr Leu Gln

            TTT GTA GAA ACT CTT GTG AAC CTT GTT AAA AAG AAG GGC AGC AAA CAG CAG TGC CTT ATG GCC TTG   869
          265 Phe Val Glu Thr Leu Val Asn Leu Val Lys Lys Lys Gly Ser Lys Gln Gln Cys Leu MET Ala Leu

            GAT ACT TTC AAA GAG TTG CTT ATC ACA GAC CTT TTG CCA GAC AAT CGG AAG CTG AGG ATT TTC AGC   935
          287 Asp Thr Phe Lys Glu Leu Leu Ile Thr Asp Leu Leu Pro Asp Asn Arg Lys Leu Arg Ile Phe Ser

            CAG CGT CCT TTT GAC AAA CTG GAA CAG TTG TCC AGT GGC AAC AAG GAC TCA AGA GAT AGA AGA CTG  1001
          309 Gln Arg Pro Phe Asp Lys Leu Glu Gln Leu Ser Ser Gly Asn Lys Asp Ser Arg Asp Arg Arg Leu

           ATA TTA TGG TAT TTT GAA CAC CAG CTG AAA CAC TTA GTG GCT GAA TTT GTG CAG GTC TTA GAA ACT  1067
          331 Ile Leu Trp Tyr Phe Glu His Gln Leu Lys His Leu Val Ala Glu Phe Val Gln Val Leu Glu Thr

            TTA AGT CAT GAT ACA TTA GTA ACC ACT AAA ACT CGA GCC CTT ACC GTG GCT CAT GAG CTG CTT TGT  1133
          353 Leu Ser His Asp Thr Leu Val Thr Thr Lys Thr Arg Ala Leu Thr Val Ala His Glu Leu Leu Cys

            AAC AAG CCT GAG GAA GAA AAG GCT CTT CTT GTG CAA GTG GTA AAT AAA CTG GGA GAT CCT CAG AAC  1199
          375 Asn Lys Pro Glu Glu Glu Lys Ala Leu Leu Val Gln Val Val Asn Lys Leu Gly Asp Pro Gln Asn

            AGA ATT GCC ACA AAA GCA TCC CAT CTG TTA GAG ACA TTA CTT TGT AAA CAT CCC AAT ATG AAA GGA  1265
          397 Arg Ile Ala Thr Lys Ala Ser His Leu Leu Glu Thr Leu Leu Cys Lys His Pro Asn MET Lys Gly

            GTT GTG TCT GGT GAA GTA GAA AGG CTA CTC TTC CGC TCA AAT ATC AGC TCC AAA GCT CAA TAT TAT  1331
          419 Val Val Ser Gly Glu Val Glu Arg Leu Leu Phe Arg Ser Asn Ile Ser Ser Lys Ala Gln Tyr Tyr

            GCA ATT TGC TTT TTA AAT CAA ATG GCT CTG TCC CAT GAA GAA AGT GAA TTG GCT AAC AAA TTA ATA  1397
          441 Ala Ile Cys Phe Leu Asn Gln MET Ala Leu Ser His Glu Glu Ser Glu Leu Ala Asn Lys Leu Ile

            ACT GTT TAC TTT TGC TTT TTT CGG ACT TGT GTC AAA AAA AAA GAT GTT GAA TCA AAA ATG CTT AGC  1463
          463 Thr Val Tyr Phe Cys Phe Phe Arg Thr Cys Val Lys Lys Lys Asp Val Glu Ser Lys MET Leu Ser

            GCC CTT TTA ACA GGT GTG AAT AGG GCA TAC CCT TAT TCC CAG ACT GGT GAT GAC AAA GTA AGG GAG  1529
          485 Ala Leu Leu Thr Gly Val Asn Arg Ala Tyr Pro Tyr Ser Gln Thr Gly Asp Asp Lys Val Arg Glu

            CAG ATT GAC ACA CTG TTT AAA GTG TTG CAT ATT GTG AAT TTT AAT ACC AGT GTC CAG GCT TTA ATG  1595
          507 Gln Ile Asp Thr Leu Phe Lys Val Leu His Ile Val Asn Phe Asn Thr Ser Val Gln Ala Leu MET
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FIG. 3. DNA and deduced amino acid sequences of CBF cDNA. The amino acid sequence corresponding to a potential nuclear localization signal, (Lys, Arg, Thr, Ala)-Lys-Lys-(Arg, Gln, Asn, Thr, Ser, Gly)-Lys (11), is underlined.

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    TTG CTT TTC CAA GTA ATG AAT TCT CAG CAG ACA ATA TCG GAT CGA TAT TAC ACA GCA TTA TAC AGG  1661
529 Leu Leu Phe Gln Val MET Asn Ser Gln Gln Thr Ile Ser Asp Arg Tyr Tyr Thr Ala Leu Tyr Arg

    AAG ATG TTG GAT CCA GGG TTG ATG ACG TGT TCC AAG CAA GCT ATG TTT CTT AAC CTT GTC TAC AAA  1727
551 Lys MET Leu Asp Pro Gly Leu MET Thr Cys Ser Lys Gln Ala MET Phe Leu Asn Leu Val Tyr Lys

    TCT CTG AAA GCT GAC ATT GTG TTG CGC CGG GTG AAG GCT TTT GTG AAG GGG TTA CTT CAA GTT ACT  1793
573 Ser Leu Lys Ala Asp Ile Val Leu Arg Arg Val Lys Ala Phe Val Lys Gly Leu Leu Gln Val Thr

    TGT CAA CAG ATG CCA CCA TTT ATA TGT GGA GCT TTA TAT CTT GTG TCT GAG ATC CTT AAA GCA AAA  1859
595 Cys Gln Gln MET Pro Pro Phe Ile Cys Gly Ala Leu Tyr Leu Val Ser Glu Ile Leu Lys Ala Lys

    CCA GGT TTA AGA AGC CAA CTA GAT GAT CAT CCG GAG TCT GAT GAT GAA GAA AAT TTT ATT GAT GCA  1925
617 Pro Gly Leu Arg Ser Gln Leu Asp Asp His Pro Glu Ser Asp Asp Glu Glu Asn Phe Ile Asp Ala

    AAT GAT GAT GAA GAC ATG GAA AAA TTC ACT GAT GCA GAC AAA GAA ACA GAG ATA GTG AAA AAA CTT  1991
639 Asn Asp Asp Glu Asp MET Glu Lys Phe Thr Asp Ala Asp Lys Glu Thr Glu Ile Val Lys Lys Leu

    GAG ACA GAG GAA ACA GTT CCT GAA ACT GAT GTA GAA ACC AAA AAA CCA GAG GTT GCT TCC TGG GTG  2057
661 Glu Thr Glu Glu Thr Val Pro Glu Thr Asp Val Glu Thr Lys Lys Pro Glu Val Ala Ser Trp Val

    CAC TTT GAT AAT TTG AAA GGT GGG AAA CAG TTA AAT AAA TAC GAT CCA TTC AGT AGA AAC CCT CTG  2123
683 His Phe Asp Asn Leu Lys Gly Gly Lys Gln Leu Asn Lys Tyr Asp Pro Phe Ser Arg Asn Pro Leu

    TTC TGT GGA GCT GAA AAT ACA AGT CTT TGG GAA CTC AAA AAG TTA TCT GTG CAT TTT CAT CCC TCC  2189
705 Phe Cys Gly Ala Glu Asn Thr Ser Leu Trp Glu Leu Lys Lys Leu Ser Val His Phe His Pro Ser

    GTG GCC CTT TTT GCA AAG ACC ATC CTT CAG GGA AAC TAT ATT CAG TAT TCA GGG GAC CCA CTG CAG  2255
727 Val Ala Leu Phe Ala Lys Thr Ile Leu Gln Gly Asn Tyr Ile Gln Tyr Ser Gly Asp Pro Leu Gln

    GAT TTC ACT CTA ATG AGA TTT TTG GAT CGA TTT GTA TAC CGA AAT CCA AAG CCC CAT AAA GGC AAA  2321
749 Asp Phe Thr Leu MET Arg Phe Leu Asp Arg Phe Val Tyr Arg Asn Pro Lys Pro His Lys Gly Lys

    GAA AAC ACA GAT AGT GTT GTG ATG CAG CCG AAA AGA AAA CAT TTT ATT AGG GAT ATT CGT CAT CTT  2387
771 Glu Asn Thr Asp Ser Val Val MET Gln Pro Lys Arg Lys His Phe Ile Lys Asp Ile Arg His Leu

    CCT GTG AAC AGT AAG GAG TTC CTT GCA AAA GAA GAA AGC CAA ATA CCA GTG GAT GAA GTG TTT TTC  2453
793 Pro Val Asn Ser Lys Glu Phe Leu Ala Lys Glu Glu Ser Gln Ile Pro Val Asp Glu Val Phe Phe

    CAC AGG TAT TAT AAA AAA GTT GCT GTT AAA GAG AAA CAA AAA CGG GAT GCA GAT GAA GAA AGT ATA  2519
815 His Arg Tyr Tyr Lys Lys Val Ala Val Lys Glu Lys Gln Lys Arg Asp Ala Asp Glu Glu Ser Ile

    GAA GAC GTG GAT GAT GAA GAA TTT GAA GAG CTG ATT GAC ACA TTT GAA GAT GAT AAC TGT TTC AGC  2585
837 Glu Asp Val Asp Asp Glu Glu Phe Glu Glu Leu Ile Asp Thr Phe Glu Asp Asp Asn Cys Phe Ser

    TCT GGA AAG GAT GAT ATG GAT TTT GCT GGA AAC GTG AAA AAG AGA ACA AAA GGA GCT AAG GAT AAC  2651
859 Ser Gly Lys Asp Asp MET Asp Phe Ala Gly Asn Val Lys Lys Arg Thr Lys Gly Ala Lys Asp Asn

    ACA TTA GAT GAA GAT TCA GAA GGT AGT GAT GAT GAA CTT GGT AAC CTG GAT GAC GAT GAA GTT TCT  2717
881 Thr Leu Asp Glu Asp Ser Glu Gly Ser Asp Asp Glu Leu Gly Asn Leu Asp Asp Asp Glu Val Ser

    TTA GGA AGT ATG GAT GAT GAA GAA TTT GCT GAA GTT GAT GAA GAT GGA GGA ACA TTC ATG GAT GTG  2783
903 Leu Gly Ser MET Asp Asp Glu Glu Phe Ala Glu Val Asp Glu Asp Gly Gly Thr Phe MET Asp Val

    TTA GAT GAT GAA AGT GAG AGC GTT CCA GAA CTT GAA GTC CAC TCC AAA GTC AGT ACT AAG AAA AGC  2849
925 Leu Asp Asp Glu Ser Glu Ser Val Pro Glu Leu Glu Val His Ser Lys Val Ser Thr Lys Lys Ser

    AAG AGA AAA GGT ACA GAT GAT TTT GAC TTT GCT GGC TCA TTT CAA GGG CCA AGA AAA AAG AAA AGA  2915
947 Lys Arg Lys Gly Thr Asp Asp Phe Asp Phe Ala Gly Ser Phe Gln Gly Pro Arg Lys Lys Lys Arg

    AAC TTA AAT GAT TCC AGC CTA TTT GTA TCT GCT GAA GAG TTT GGC CAT CTA TTG GAT GAA AAT ATG  2981
969 Asn Leu Asn Asp Ser Ser Leu Phe Val Ser Ala Glu Glu Phe Gly His Leu Leu Asp Glu Asn MET

    GGA TCC AAG TTT GAT AAC ATT GCA TGA ATG CCA TGG CTA ACA AAG ATA ATG CAA GTC TCA AAC AGC  3047
991 Gly Ser Lys Phe Asp Asn Ile Ala TER

    TTA GAT GGG AGG CTG AAC GTG ATG ACT GGC TAC ACA ACA GAG ATG CAA AAA GTA TCA TCA AGA AAA  3113
    AGA AAC ATT TTA AAA AGA AGA GGA TTA AAA CCA CTC AAA AAA CTA AAA AAC AAA GGA AAT GAG TTA  3179
    TTA ATG TAA ATT ATA GAT TAA ATT TCT ACT TAC ATC TAA AAA AAA AAA AAA AAA AAA AAA AA   3244
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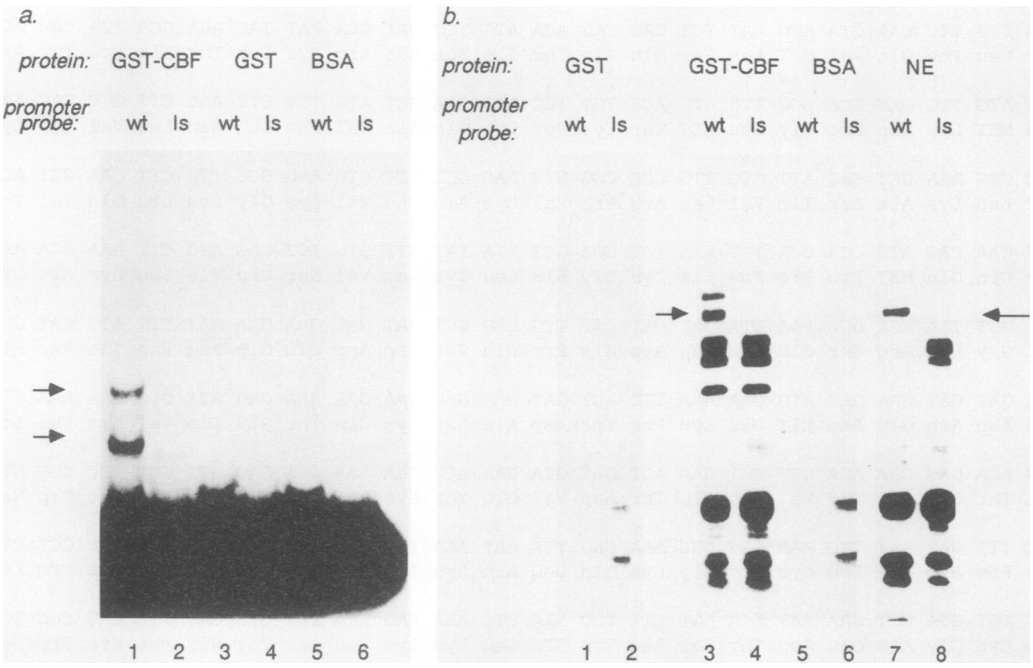

FIG. 4. Sequence-specific binding activity of GST-CBF produced in bacteria. Shown are results of gel mobility shift (a) and exonuclease III protection (b) assays of wt or ls 73/63 promoter probes incubated with 100 ng of bacterially produced GST-CBF or GST, each supplemented with 8 μg of bovine serum albumin (BSA), 8 μg of bovine serum albumin alone, or 10 μg HeLa cell nuclear extracts (NE). The promoter probes extend from positions −100 to +60 of the hsp70 promoter and are labeled at the *Hin*fI site at +60. Arrows indicate the sequence-specific shifted species (a) and the specific protection from exonuclease III at position −76 of the hsp70 promoter (b).

3006, and a 3′ nontranslated region of 208 nucleotides followed by a poly(A) tail (Fig. 3). The composite clone may represent the complete coding region, since the product from hybrid-selected in vitro translation of HeLa cell RNA migrated on SDS-PAGE with a mobility similar to that produced from in vitro translation of T7 RNA polymerase-transcribed CBF cRNA (Fig. 2c) as well as to CBF synthesized in vivo from mammalian cells transfected with CBF cDNA (see Fig. 5a). The predicted protein sequence of 114 kDa, however, is much less than the molecular mass observed in vitro and in vivo. This discrepancy may result from aberrant migration or posttranslational modifications.

The putative amino acid sequence of CBF contains a consensus nuclear localization signal (residues 943 to 947), which may be a requirement for a protein of this size to enter the nucleus. Analysis of the CBF protein sequence also reveals potential sites of modification, including one tyrosine kinase phosphorylation site (residue 761), four cAMP-cGMP-dependent kinase phosphorylation sites (residues 278, 720, 874, and 951), and numerous protein kinase C and casein kinase II phosphorylation sites. The predicted amino acid sequence of CBF is distinct from those of the two previously cloned CCAAT-binding factors CTF and C/EBP, and a search of the GenBank data base failed to identify any related proteins. The CBF polypeptide is predicted by Chou-Fasman Analysis to be predominantly α helical and by Kyte-Doolittle Analysis to be predominantly hydrophilic except for a slightly hydrophic domain between residues 500 and 650. Interestingly, within this region lies a potential amphipathic α helix in which 11 of 22 amino acids are identical to those of the sigma homology domain of yeast TFIID (16). This region may represent a conserved domain involved in protein-protein interactions among transcription factors.

CBF cDNA was subcloned into the pGEX bacterial expression vector (33) to generate a fusion protein with the 26 kDa of the GST protein. GST-CBF fusion protein was purified and assayed for sequence-specific binding activity by electrophoretic mobility shift assays. The two shifted species observed with GST-CBF were specific for the CCAAT element of the hsp70 promoter, since they were detected only with the wt promoter probe, not with the ls promoter probe (Fig. 4a, lanes 1 and 2). We do not know the relationship of the two shifted species; they may represent monomeric versus dimeric complexes, or they may result from partial proteolysis of GST-CBF. The binding activity of GST-CBF resides within the CBF moiety, since GST alone does not bind to the promoter probes (Fig. 4a, lanes 3 and 4). The GST-CBF/hsp70 promoter complexes migrated faster than the complex formed with HeLa cell nuclear extracts, suggesting that the latter may be composed of additional heterologous subunits. Alternatively, the difference in mobility may result from modifications of CBF in mammalian cells. Binding of GST-CBF to the hsp70 promoter probe resulted in two exonuclease III-protected fragments with termini at positions −80 and −76, which were lost when the wt probe was substituted with the ls probe (Fig. 4b, lanes 3 and 4). Position −76 corresponds to the boundary previously detected in HeLa cell nuclear extracts (40). As with the gel shift assays, the binding activity resided with CBF, since the GST moiety alone did not protect these fragments from exonuclease III digestion. These assays confirm the sequence-specific binding activity of the polypeptide encoded by the CBF cDNA clone.

Cotransfected CBF cDNA stimulates transcription from the hsp70 promoter in a CCAAT-dependent manner. The effect of CBF on transcriptional activity of target promoters was monitored by cotransfection in COS cells. CBF cDNA was

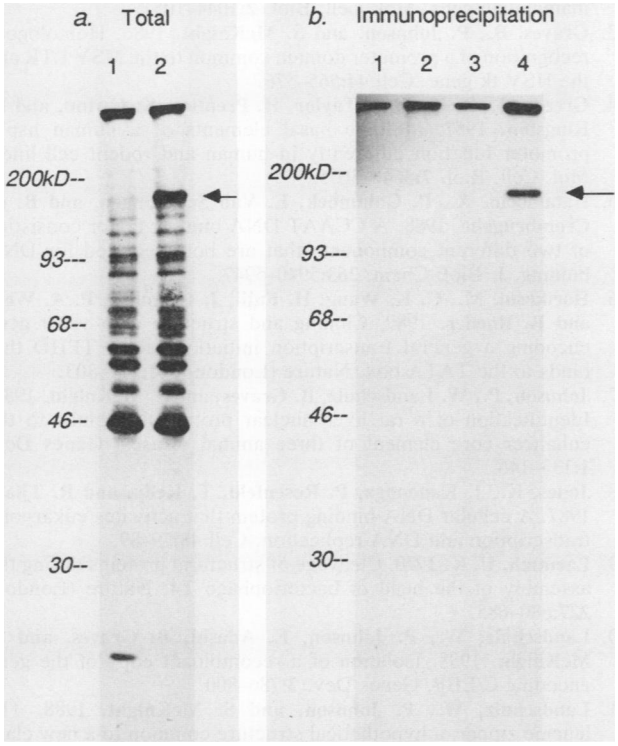

FIG. 5. Expression of CBF in transfected mammalian cells. (a) SDS-PAGE of total lysates prepared from metabolically labeled COS cells transfected with pMT2 (lane 1) or pMT2-CBF (lane 2). (b) SDS-PAGE of immunoprecipitated material prepared from lysates of metabolically labeled COS cells transfected with pMT2 (lanes 1 and 2) or pMT2-CBF (lanes 3 and 4). Immunoprecipitations were conducted with preimmune serum (lanes 1 and 3) or with antiserum (lanes 2 and 4) raised against bacterially produced GST-N1/534. A CBF-related polypeptide of similar mobility was observed in lane 2 upon prolonged autoradiographic exposure.

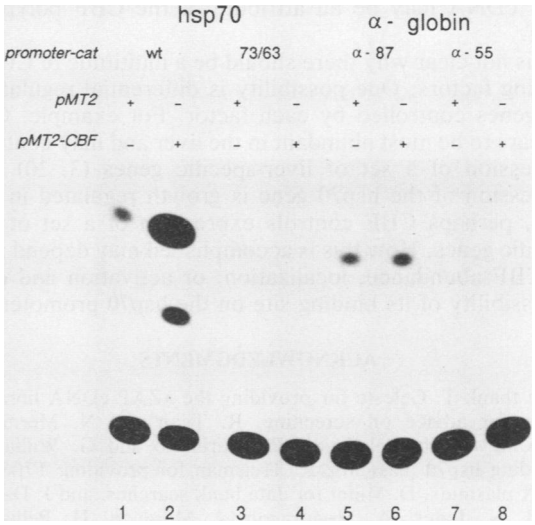

FIG. 6. Stimulation by CBF of transcription from the hsp70 promoter. Enzymatic assays of CAT activity in extracts prepared from COS cells cotransfected with the expression vector pMT2 or with pMT2-CBF and the following promoter-CAT reporter genes: the wt hsp70 promoter from positions −100 to +150 (wt); an ls mutation in the CCAAT element of the hsp70 promoter (73/63); the α-globin promoter from positions −87 to +36, containing a CCAAT element (α-87); and the α-globin promoter from positions −55 to +36, deleting the CCAAT element (α-55).

subcloned into pMT2, a mammalian polycistronic expression vector containing the simian virus 40 (SV40) origin of replication, SV40 enhancer, and adenovirus major late promoter (4). To confirm that CBF was synthesized, radiolabeled polypeptides from transfected cells were examined by SDS-PAGE and autoradiography. A polypeptide of molecular mass greater than 116 kDa was seen in extracts prepared from pMT2-CBF-transfected cells but not in extracts prepared from cells transfected with pMT2 (Fig. 5a). This polypeptide was recognized by antiserum produced against GST-N1/534 fusion protein containing the N-terminal 534 residues of CBF (Fig. 5b, lane 4).

Two cellular (hsp70 and α-globin) and two viral (SV40 early and Rous sarcoma virus [RSV]) promoters were examined for responsiveness to CBF. These promoters were fused to a CAT reporter gene, allowing both enzymatic assay of the CAT protein product and S1 nuclease protection assay of CAT mRNA. Cotransfection with pMT2-CBF enhanced the promoter activity of the wt hsp70 promoter five- to sevenfold by CAT assay (Fig. 6, lanes 1 and 2) and four- to sixfold by S1 nuclease protection (data not shown). Activation of the hsp70 promoter was dependent on the CCAAT element since a mutation that disrupted the CCAAT-binding site was nonresponsive (Fig. 6, lanes 3 and 4). Although the α-globin promoter contains a CCAAT element that is of functional importance (Fig. 6, lanes 5 and 7; 30), it was nonresponsive to cotransfected pMT2-CBF (Fig. 6, lanes 5 and 6). The activities of the SV40 early and the RSV promoters were also not significantly altered by cotransfected pMT2-CBF (data not shown). The stimulatory activity provided in *trans* by cotransfected CBF cDNA was not seen when either an antisense or a truncated construct of CBF, encoding residues 1 to 710, was used (data not shown).

DISCUSSION

Several distinct CCAAT-binding activities in eucaryotes have been described (6–8, 15, 17, 29). CTF (18, 27), CP1 (6, 8), and now CBF have been shown to bind in vitro to the CCAAT element of the hsp70 promoter. CTF and CP1 are reported to be antigenically unrelated (26), and as shown here, CBF and CTF are distinct by deduced protein sequence. The relationship between CBF and CP1 remains to be examined. Bacterially produced CTF (30) and C/EBP (20) are able to bind DNA without accessory proteins, while CP1 and CP2, purified from HeLa cells (6), are composed of heterologous subunits which are necessary for DNA-binding activity. Several lines of evidence in this study suggest that CBF is able to bind DNA without accessory proteins. However, it is possible that accessory factors serve to stabilize or increase the affinity or specificity of binding or are essential for transcriptional activity.

Cotransfected CBF cDNA stimulated transcription from the hsp70 promoter but not from the α-globin or the RSV promoter, both of which contain CCAAT elements. The α-globin promoter binds CTF and, when assayed in *Drosophila* cells, is stimulated by cotransfected CTF cDNA (30). The α-globin, RSV, and hsp70 promoters all bind CP1 (6) or a CP1-like activity (8) in vitro. Perhaps CBF does recognize the α-globin and RSV promoters, but transcriptional stimulation requires additional factors that are not present or of sufficient abundance in COS cells. Alternatively, the specificity for the hsp70 promoter displayed by cotransfected

CBF cDNA may be an attribute of the CBF polypeptide itself.

It is not clear why there should be a multitude of CCAAT-binding factors. One possibility is differential regulation of the genes controlled by each factor. For example, C/EBP appears to be most abundant in the liver and may control the expression of a set of liver-specific genes (3, 10). Since expression of the hsp70 gene is growth regulated in HeLa cells, perhaps CBF controls expression of a set of G1/S-specific genes. How this is accomplished may depend in part on CBF abundance, localization, or activation and on the accessibility of its binding site on the hsp70 promoter.

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