Cell Cycle-dependent Switch of Up- and Down-regulation of Human hsp70 Gene Expression by Interaction between c-Myc and CBF/NF-Y*

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A CCAAT box-binding protein subunit, CBF-C/NF-YC, was cloned as a protein involved in the c-Myc complex formed on the G1-specific enhancer in the human hsp70 gene. CBF-C/NF-YC directly bound to c-Myc in vitro and in vivo in cultured cells. The CBF/NF-Yc-Myc complex required the HSP-MYC-B element as well as CCAAT in the hsp70 G1-enhancer, while the purified CBF subunits recognized only CCAAT even in the presence of c-Myc. Both the HSP-MYC-B and CCAAT elements were also required for the enhancer activity. In transient transfection experiments, the CBF/NF-Yc-Myc complex, as well as transcription due to the G1-enhancer, was increased by the introduction of c-Myc at low doses but decreased at high doses. The repression of both complex formation and transcription by c-Myc at high doses was abrogated by the introduction of CBF/NF-Y in a dose-dependent manner. Furthermore, the CBF/NF-Yc-Myc complex bound to the G1-enhancer appeared in the early G1 phase of the cell cycle when c-Myc was not highly expressed and gradually disappeared after the c-Myc expression reached its maximum. The results indicate that the cell cycle-dependent expression of the hsp70 gene is regulated by the intracellular amount of c-Myc through the complex formation states between CBF/NF-Y and c-Myc.

The human heat shock 70 (hsp70) gene is induced by various stresses, including heat shock (for reviews, see Refs. 1–3). Without stress, the hsp70 gene is expressed in a cell cycle-dependent manner, namely in the G1 and the S phases (4–7). In addition to the heat shock element, several transcriptional regulatory elements, including the sites for Sp1, AP2, ATF, and CTF, have been found to contribute to a basal level of hsp70 gene expression in the hsp70 gene promoter region. CTF is a CCAAT box-binding protein, and two CCAAT boxes are present in the hsp70 gene promoter, at about −150 and −90 from the transcription start site (8). Not only CTF (9) but also CBF of 114 kDa (10) have been cloned as binding proteins to these CCAAT sequences. The hsp70 gene expression is also induced by several oncogenes, including c-myc (11, 12), c-myb, p53, T antigens of SV40 and polyomavirus (13), and adenovirus E1A (14–17). Although the precise mechanisms of the regulation by these oncogenes have not been clarified, p53, Myb, and E1A have been reported to regulate the hsp70 gene expression by interacting with other proteins that directly bind to the respective transcriptional elements in the hsp70 gene promoter (18). Both E1A and p53 competitively bind to the CBP of 114 kDa to stimulate and repress the hsp70 promoter activity (19). Up-regulation of the gene by c-myc has been reported in Drosophila and humans, and the region involved in the regulation was identified to be from −120 to −1,250 in the human hsp70 promoter (11, 12). We have identified two sequences bound by c-Myc complexes in this region and termed them HSP-MYC-A (from −232 to −226) and HSP-MYC-B (from −157 to −151) (20). HSP-MYC-B is adjacent to an inverted CCAAT box and one nucleotide is overlapped. Both HSP-MYC-A and HSP-MYC-B functioned as an origin of DNA replication, and the plasmids carrying both or either of the sequences worked as an autonomous replicating plasmid (21). Of the two sequences, only HSP-MYC-B showed a transcriptional enhancer activity which contributed to the G1-specific expression of hsp70 in the cell cycle (7). This enhancer function of HSP-MYC-B was confirmed by another group (22). The c-Myc complex was therefore thought to be involved in the cell cycle-dependent expression of the hsp70 gene. In this study, we have verified the association of c-Myc with CBF/NF-Y, CCAAT-binding proteins, and the involvement of the c-Myc-CBF/NF-Y complex formed on the HSP-MYC-B element in the regulation of hsp70 expression.

EXPERIMENTAL PROCEDURES

Plasmids—pACT-CBF-C was obtained from one-hybrid plasmids followed by two-hybrid screening targeting the HSP-MYC-B and C-Myc binding activities. For pCMV-CBF-C, pGEX-CBF-C, and pMBP-CBF-C, the EcoRI-Xhol fragment of pACT-CBF-C was inserted into the EcoRI-Xhol site of pcdNAS (Invitrogen), pGEX-5X-1 (Amersham Pharmacia Biotech), and pMAL-6P-I,† respectively. For pACT-CBF-A and -B, the NcoII-Xhol fragment of pCite-2a-CBF-A and -B, respectively (23), was inserted into the respective sites in pACTZ. The pCMV-CBF-A, -B, pGEX-CBF-A, -B, and pMBP-CBF-A, -B of the BglII-Xhol fragment of pACT-CBF-A, -B was inserted into the BamHI-Xhol site of pcDNA3, pGEX-5X-1, and pMAL-6P-I, respectively, pCMV-GAL4-CBF-A, B, C of the HindIII-BamHI fragment of pGBT9 (CLONTECH) and BglII-Xhol fragment of pACT-CBF-A, B, and C, respectively, were inserted into the HindIII-Xhol site of pcdNAS. The c-Myc plasmids for the mammalian two-hybrid system, wild type, N, Δ177, ΔM, and ΔZip, have been described previously (24), and ΔH1 and ΔLZ were constructed according to the published procedure (25). pGEX-CBF-C (EP) (pGEX-CBF-C) was digested with PstI and the resultant large fragment was self-ligated. pGEX-CBF-C/EN (pGEX-CBF-C) was digested with

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The nucleotide sequences of HSM-1/NF-YC and HSM-2 reported in this paper have been submitted to the GenBankTM/EBI Data Bank with accession numbers D85425 and D89986, respectively.

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NcoI and XhoI, and the resultant large fragment was treated with the Klenow fragment followed by self-ligation. pGEX-CBF-C(NX), the pGEX-CBF-C, was digested with NcoI and the resultant large fragment was self-ligated. pGEX-CBF-C(NP), pGEX-CBF-C(NP), was digested with NcoI and the resultant large fragment was self-ligated. pGEX-CBF-C(FP), the CBF-C(FP) cDNA, was digested with NcoI and the resultant large fragment was self-ligated. pGEX-CBF-C(FP), the CBF-C(FP) cDNA, was digested with NcoI and the resultant large fragment was self-ligated.

The proteins recovered from the resin were separated in a 7.5% polyacrylamide gel containing 4 M urea and subjected to blotting to a nitrocellulose filter. The filter was incubated with rabbit anti-CBF-C/NF-Y antibody or a mouse anti-c-Myc antibody (C33, Santa Cruz), respectively. The nitrocellulose filter was then incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized using ECL (Amersham Pharmacia Biotech). The absorbance was measured at 492 nm.

The results of the gel-electrophoresis were as follows: the proteins recovered from the resin were separated in a 7.5% polyacrylamide gel containing 4 M urea and subjected to blotting to a nitrocellulose filter. The filter was incubated with rabbit anti-CBF-C/NF-Y antibody or a mouse anti-c-Myc antibody (C33, Santa Cruz), respectively. The nitrocellulose filter was then incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized using ECL (Amersham Pharmacia Biotech). The absorbance was measured at 492 nm.

RESULTS AND DISCUSSION

Cloning of cDNA Encoding a Protein That Recognizes Together with c-Myc the Sequence Containing HSP-MYC-B and CCAAT—The c-Myc-containing complex formed on the HSP-MYC-B element was suggested to be responsible for the G1-specific enhancer activity of the element, but c-Myc did not directly bind to the element (20). We thus planned to first clone proteins that directly bind to HSP-MYC-B, and then to select one that associates with c-Myc. Since the wtB oligonucleotide, 5'-CTGGCCTCTGAGTGG-3', containing the HSP-MYC-B showed a strong enhancer activity (Ref. 12; Fig. 9), tetramerized wtB was used as a targeting sequence for the one-hybrid screening. After introducing pHISi-4XwtB into S. cerevisiae to integrate in yeast genome, the human brain cDNA library in pACT2 was applied to the transformant, and the yeast were cultured in His(-) media. Among the approximately 4 \times 10^6 clones tested, more than 100 His-positive clones were obtained and subjected to \beta-galactosidase assays. Plasmid DNAs were extracted from the 12 \beta-galactosidase-positive clones and used for re-transforming the S. cerevisiae SFY526 previously transformed with pGBT9-c-myc (30). The double transformants surviving in His(-) media were then tested for \beta-galactosidase activity to see the interaction with c-Myc fused to the GALBD.

Finally, 12 clones were obtained and these were classified into two groups, tentatively named as HSM-1 and HSM-2. Through the BLAST search of their nucleic acid sequences, HSM-1 was identified as a human homologue of rat NF-YC/CBF-C (31), while HSM-2 was a novel gene that encodes a protein with slight homology to NF-YC/CFB-C. We further characterized HSM-1, i.e. the human CBF-C/NF-YC, in this study.

**CBF/NF-Y** is a CCAAT-binding protein with three subunits of CBF, CBF-A, CBF-B, and CBF-C, which correspond to NF-YB, NF-YA, and NF-YC, respectively. The rat, mouse, and human cDNAs for CBF-A, CBF-B, and CBF-C cDNAs have already been cloned (31-37). Among the three subunits of CBF/NF-Y, CBF-B/NF-YA directly recognizes the CCAAT sequence, not by itself but in the complex of the three subunits (23, 38, 39). The cDNA for human CBF-C/NF-Y was cloned in this study as HSM-1 in the screening of HSP-MYC-B-binding proteins, probably because the wtB probe used in the one-hybrid screening contained an inverted CCAAT in addition to the HSP-MYC-B element, and yeast homologues of CBF-B/NF-YA and CBF-A/NF-YB (HAP-2 and HAP-4, respectively) may complement the respective human homologues to sustain the binding activity of the cloned CBF-C/NF-YC to the wtB probe as described previously (40). The HSM-1 cDNA we cloned contained 1,381 nucleotides, with several variations compared with those in the human CBF-C/NF-YC cloned by Bellerini et al. (41), and encoded exactly the same 335 amino acids as the human CBF-C/NF-YC. As in the case of CBF-A/NF-YB and...
CBF-B/NF-YA, only two amino acids were different between human and rat CBF-C. The expression of human CBF-C/NF-YC in human tissues was examined by Northern blot analyses. The results revealed that CBF-C/NF-YC was expressed almost ubiquitously in all the human tissues tested (data not shown), as reported previously (41).

**Interaction of CBF/NF-Y with c-Myc in Vitro**—To verify the interaction between CBF-C/NF-YC and c-Myc, in vitro binding assays were carried out using CBF-C purified as fused to glutathione S-transferase (GST) and the human Raji cell extract containing c-Myc. Various deletion mutants of CBF-C as well as the wild type protein were expressed in *E. coli* as GST fusion proteins and purified. GST-c-Myc was then treated with PreScission protease to release a free recombinant c-Myc. The free c-Myc was incubated with either MBP by itself or fusion proteins to CBF-A, CBF-B, CBF-C, or Max, and then precipitated with an anti-c-Myc antibody (C-33, Santa Cruz) or nonspecific IgG. The precipitates were separated in a 10% polyacrylamide gel containing SDS and blotted with an anti-MBP antibody (New England Biolabs). My and Ig indicate the anti-c-Myc antibody and nonspecific IgG, respectively. In indicates the lanes containing either a fusion protein or MBP.

Similar binding assays were carried out using antibodies against each subunit of CBF/NF-Y. Since the amino acid sequences of both CBF-A/NF-YB and CBF-B/NF-YA are identical in rat and human except for 2 amino acids, we used rat cDNAs for CBF-A and CBF-B (36, 37), instead of the corresponding human cDNAs. Three subunits of CBF, as well as Max as a positive control interacting with c-Myc, were expressed in *E. coli* as fusion proteins to MBP and purified by the affinity column containing maltose. MBP-c-Myc was first expressed in *E. coli* and purified by the maltose resin. The recombinant full-length c-Myc was then obtained by releasing MBP with PreScission protease and subjected to binding reactions. The full-length c-Myc bound to the E-box sequence together with Max (characterization of the recombinant c-Myc in full-length will be described elsewhere). The MBP fusion protein with either CBF subunits or Max, or MBP alone, was mixed with c-Myc and applied to the agarose resin coupled with a mouse anti-c-Myc monoclonal antibody (C-33, Santa Cruz) or nonspec-
Specific mouse IgG. The proteins bound or not to either of the antibody-coupled resins were analyzed by Western blotting with an anti-MBP antibody (Fig. 1B). MBP-Max was recovered from the anti-c-Myc antibody-coupled resin (Fig. 1B, lane 1) but not from the resin with nonspecific IgG (Fig. 1B, lane 2). Neither of the resins bound free MBP (Fig. 1B, lanes 13 and 14). A little recovery of MBP-CBF-A from both resins indicated that CBF-A nonspecifically interacts with the resin (Fig. 1B, lanes 4 and 5). MBP-CBF-C was detected in the eluate from the resin with the anti-c-Myc antibody but not with IgG (Fig. 1B, lanes 10 and 11), while MBP-CBF-B was hardly recovered from either resin (Fig. 1B, lanes 7 and 8). The results indicate that CBF-C/NF-YC directly binds to c-Myc in a specific manner.

Interaction of CBF/NF-Y with c-Myc in Vivo—To examine the association of CBF/NF-Y with c-Myc in vivo, a mammalian two-hybrid assay was carried out. The wild type or various deletion mutants of c-Myc were fused to the VP16 transcriptional activation domain (VP16AD). MBP-Max was recovered from the anti-c-Myc antibody-coupled resin (Fig. 1B, lane 1) but not from the resin with nonspecific IgG (Fig. 1B, lane 2). Neither of the resins bound free MBP (Fig. 1B, lanes 13 and 14). A little recovery of MBP-CBF-A from both resins indicated that CBF-A nonspecifically interacts with the resin (Fig. 1B, lanes 4 and 5). MBP-CBF-C was detected in the eluate from the resin with the anti-c-Myc antibody but not with IgG (Fig. 1B, lanes 10 and 11), while MBP-CBF-B was hardly recovered from either resin (Fig. 1B, lanes 7 and 8). The results indicate that CBF-C/NF-YC directly binds to c-Myc in a specific manner.

Interaction of CBF/NF-Y with c-Myc in Vivo—To examine the association of CBF/NF-Y with c-Myc in vivo, a mammalian two-hybrid assay was carried out. The wild type or various deletion mutants of c-Myc were fused to the VP16 transcriptional activation domain (VP16AD), and each subunit of CBF/NF-Y was fused to the GALBD. The constructs were transfected to hamster CHO cells in various combinations together with pHIE17-MX-Luc, which contains 6xGAL4, and HTLV-1 promoter linked to the luciferase gene and luciferase assays were carried out (Fig. 2). With the wild type c-Myc, CBF-A and CBF-C stimulated luciferase activity, while CBF-B did not. c-Myc thus interacted in vivo with CBF-C, but not with CBF-B, in transfected CHO cells as well as in vitro. CBF-A was also suggested to interact with c-Myc in vivo, less strongly than CBF-C. The affinity difference between CBF-A and CBF-C was more significant in the interaction with c-Myc deletion mutants, as described below. Among the various c-Myc mutants examined, Δ177 and ΔM deleting both myc boxes-1 and -2 or the myc box-2 followed by the internal region, respectively, interacted strongly with CBF-C and weakly with CBF-A, but did not interact with CBF-B. Deletions in the C-terminal region of c-Myc, not only a big deletion in mutant N but also small deletions in mutants ΔHZ, ΔH1, and ΔZip, resulted in the loss of CBF binding activity. These results implied that the C-terminal region of c-Myc spanning amino acids 368–435 is required for the interaction with CBF-C/NF-YC, and additionally with CBF-A/NF-YB.

The in vivo association between c-Myc and CBF/NF-Y was also examined by co-immunoprecipitation assays. A rabbit polyclonal anti-CBF-C antibody was prepared by GST-CBF-C as an immunogen. The antibody specifically detected CBF-C but not CBF-A or CBF-B in human, mouse, and rat cells (data not shown). The human Raji cell extract was first precipitated with the antibody against CBF-A, CBF-B (Rockland Inc.), or
CBF-C, or with nonspecific IgG. The precipitates were separated in an SDS-polyacrylamide gel and blotted with an anti-c-Myc antibody (C-33, Santa Cruz) (Fig. 3). The 64-kDa c-Myc in the Raji extract was precipitated with the antibody against CBF-A, CBF-B, or CBF-C (Fig. 3, lanes 3–5), while the precipitate with nonspecific IgG did not contain the protein reacting with the anti-c-Myc antibody (Fig. 3, lane 2). The results indicated that c-Myc associates with all CBF subunits in vivo in human Raji cells. Since the three CBF subunits form a stable complex in vivo (23, 38, 39), c-Myc was thought to associate with the CBF/NF-Y complex in vivo.

DNA-Protein Complex Targeting HSP-MYC-B and CCAAT Sequences—We have previously shown that a protein complex containing c-Myc bound to the HSP-MYC-B sequence (20). To assess the involvement of CBF/NF-Y in the c-Myc complex on HSP-MYC-B, bandshift assays were carried out using specific antibodies. The Balb 3T3 nuclear extract was treated with an antibody against c-Myc (OM11–905, Genosys), CBF-A, -B, or -C, prior to binding reactions with a labeled wtB oligonucleotide, and separated in a polyacrylamide gel (Fig. 4). In the absence of antibodies, two specific DNA-protein complexes, complexes I and II, were detected (Fig. 4, A and B, lane 2). The anti-c-Myc antibody abrogated the formation of both complexes, while neither complex was affected by IgG (Fig. 4, A, lanes 3–6). All three antibodies against CBF subunits impaired the formation of complex I but not of complex II (Fig. 4, B, lanes 5–10). CBF/NF-Y was thus suggested to be involved in c-Myc containing complex I on the wtB oligonucleotide, but not in complex II. A rabbit polyclonal anti-Max antibody (C-124, Santa Cruz) did not interfere with DNA-protein complex formation (data not shown). Since c-Myc by itself does not bind to the wtB probe, complex I may interact with the sequence via CBF/NF-Y. Complex II may contain an unidentified protein(s) other than CBF/NF-Y between c-Myc and the wtB sequence.

To determine the nucleotides recognized by complexes I and II, binding reactions were carried out in the presence of various...
mutants of the wtB probe (Fig. 4C). Mutations were introduced to wtB (WW) within CCAAT (Wm) or HSP-MYC-B (mW), or both (mm). Another variant, D, in which CCAAT was deleted from wtB, was also constructed. Excess amounts of nonlabeled wtB oligonucleotide (WW) abolished the specific nucleoprotein complexes (Fig. 4C, lanes 2–4). None of the wtB variants (mW, Wm, or mm), on the other hand, affected the formation of the complexes (Fig. 4C, lanes 5–16). The results suggested that not only the inverted CCAAT but also the adjacent nucleotides in the HSP-MYC-B element were involved in DNA-protein association of complexes I and II.

When the CBF/NF-Y proteins purified as MBP fusion proteins were subjected to binding reactions with the labeled wtB probe, a single nucleoprotein complex appeared, with a faint background due to degraded proteins (Fig. 4D, lane 3). The DNA-protein complex of the purified CBF/NF-Y protein on wtB was canceled by excess amounts of nonlabeled oligonucleotide carrying the intact CCAAT (WW and mW; Fig. 4D, lanes 5–12) but not by those with mutations or deletion in CCAAT (Wm, ΔC and mm; Fig. 4D, lanes 8–10). Thus, the purified CBF/NF-Y proteins recognized the CCAAT motif in wtB, while complex I containing the CBF proteins and c-Myc were formed on both HSP-MYC-B and CCAAT. The results of methylation interference experiments also suggested that complex I was formed on almost the whole length of wtB sequence, while complex II and the complex of recombinant CBF proteins interacted with the probe within either the HSP-MYC-B or the CCAAT element, respectively (data not shown).

Although c-Myc was involved in complex I, the purified c-Myc (the MBP-released recombinant protein as described above for Fig. 1B) did not recognize the wtB probe by itself (Fig. 5A, lanes 2–4). The addition of the purified c-Myc did not affect the nucleoprotein complex formed between CCAF in the wtB sequence and the purified CBF proteins; complex I observed in the nuclear extract was not reconstituted by the purified CBF proteins and the purified c-Myc (Fig. 5A, lanes 8–16). Complex I, representing a complex in vivo, was thus thought to contain other proteins in addition to the CBF proteins and c-Myc.

We have shown above that c-Myc directly associates with CBF-C (Fig. 1B). A previous report revealed that CBF-A and CBF-C were first associated and that CBF-B possessing the DNA binding activity was recruited to the precomplexed CBF-A and CBF-C (23, 42). CBF-A and c-Myc may independently interact with different sites of CBF-C, or alternatively CBF-A and c-Myc may competitively associate with CBF-C. Since the CBF complex on CCAAT was neither decreased nor
shifted by the addition of c-Myc (Fig. 5A), it was thought that c-Myc was unable to interact with CBF-C in the preassociated CBF complex on CCAAT. c-Myc was therefore incubated with CBF-A, -B or -C prior to the addition of the other two subunits and the wtB probe (Fig. 5B). When CBF-B and -C were added to the preincubation of c-Myc and CBF-A, the CBF complex on CCAAT was formed as efficiently as in the absence of c-Myc (Fig. 5B, lanes 1–5). The affinity of CBF-C to CBF-A was therefore thought to be stronger than that to c-Myc. The addition of CBF-A and CBF-C to the preincubated CBF-B with c-Myc also yielded the CBF complex as in the absence of c-Myc (Fig. 5B, lanes 6–10), although CBF-C preferred CBF-A to c-Myc. The preincubation of CBF-C with c-Myc, on the other hand, greatly inhibited the formation of the CBF complex on CCAAT in the wtB probe (Fig. 5B, lanes 11–15).

No bands due to nucleoprotein complexes other than the CBF complex alternatively appeared. The results suggested that the preceding interaction between CBF-C and c-Myc showed priority to CBF-A, which has a higher affinity to CBF-C. The disruption of the CBF complex on CCAAT did not yield another nucleoprotein complex on wtB as it was thought that an unidentified protein (or proteins) in the nuclear extract, other than c-Myc and the CBF proteins, is involved in complex I on wtB and that the protein (or proteins) mediates between the complex and wtB sequence. It was therefore thought that c-Myc interferes with the association of CBF-C with CBF-A when c-Myc precedes CBF-A in the interaction with CBF-C, but that it cannot intrude into the precomplexed CBF proteins.

**Regulation of the Transcriptional Activity of wtB Containing HSP-MYC-B and CCAAT by c-Myc**—We have previously reported that the HSP-MYC-B element was required for G1-specific enhancer activity of the wtB sequence (7). Since the wtB sequence contains CCAAT besides HSP-MYC-B, the effects of CCAAT on the transcriptional activity of wtB were examined. The wild type (WW) or wtB variant with mutations within either CCAAT (Wm) or HSP-MYC-B (mW), or both (mm), or deletion of CCAAT (D) (see Fig. 4), was ligated to the TATA box of the human hsp70 gene linked to the luciferase gene, and transfected to mouse Balb 3T3 cells. The results of the luciferase activity assays of the cells are shown in Fig. 6. The wild type wtB (WW) in pwtB-TATA-Luc stimulated luciferase activity 22-fold of that due to pHS-TATA-Luc containing.

**Fig. 7.** Effect of additional c-myc expression on the transcriptional activity of the wtB sequence and the specific complex formation on the wtB sequence. A, various amounts of a c-myc expression vector, either in sense (pEF-c-myc) or anti-sense (pEF-anti-c-myc) orientation (51), were transfected into Balb 3T3 cells together with the reporter plasmid pwtB-TATA-Luc. Two days after transfection, the luciferase activities were assayed. Relative luciferase activities to that due to the reporter plasmid alone are shown. B, Balb 3T3 cells were transfected with various combinations of expression vectors for the CBF subunits, as indicated, in addition to 1 µg of pEF-c-myc and the reporter pwtB-TATA-Luc. Two days after transfection, the luciferase activities were assayed. Relative luciferase activities of the reporter plasmid alone are shown. C and D, Balb 3T3 cells were transfected with various amounts of the c-myc expression vector pEF-c-myc as in A. Two days after transfection, the nuclear extracts were prepared from the cells and subjected to bandshift assays using a labeled wtB (C) or Sp1 element (D) as a probe. The positions of the specific CBF/NF-Y complexes I and II, the Sp1 complex, and the free probe are indicated as I, II, Sp1, and F, respectively. E, aliquots of the same nuclear extracts prepared in C and D were also analyzed by Western blotting using the anti-c-Myc antibody (C33) or an anti-CBF-C antibody.
transcriptional activity of the wtB sequence during the cell cycle that of the cells at random culture (described previously (12)). The luciferase activity was assayed at various times after transfection of the reporter plasmid pwtB-TATA-Luc, pmWTATA-Luc, pmm-TATA-Luc, or pWT-3'-UTR-Luc, which have the TATA box alone. All the mutants tested lost the enhancer activity. The luciferase activity of pmWTATA-Luc, pmm-TATA-Luc, pmm-TATA-Luc, or pWT-3'-UTR-Luc was comparable to that of pHs-TATA-Luc. The results suggested that only the HSP-MYC-B element but also the CCAAT motif are required for the transcriptional enhancer activity. Complex I, recognizing both HSP-MYC-B and CCAAT, but not the CBF complexes targeting only CCAAT, was therefore implied to be responsible for the transcriptional activity. Complex II which did not contain CBF proteins was also suggested to be involved in the regulation of transcriptional activity, since the complex was formed on both HSP-MYC-B and CCAAT.

Since c-myc protein was shown to be involved in both complexes I and II, the effects of c-Myc on the transcriptional activity were examined. Various amounts of the expression vector for c-Myc either in sense (pEF-c-myc) or antisense orientation (pEF-anti-c-myc) were transfected to Balb 3T3 cells together with pWT-3'-UTR-Luc, and the luciferase activities were assayed (Fig. 7A). While the c-myc construct in antisense did not affect the luciferase activity at all, the sense construct yielded a biphasic fluctuation of enzyme activity (pEF-c-myc stimulated the luciferase activity in a dose-dependent manner to a peak of 1.8-fold at 10 ng, but higher doses repressed the activity). When 1 μg of pEF-c-myc was co-transfected, luciferase activity was about 40% of that in the absence of pEF-c-myc. The 40% activity thus repressed by 1 μg of pEF-c-myc was restored to the original level by the co-transfected expression vectors for both CBF-A and CBF-C or the three CBF subunits (Fig. 7B). CBF-C, which directly associates with c-Myc to form the core of complex I, did not release itself by the repression by pEF-c-myc. Neither CBF-A nor CBF-B affected the repressed activity. The results suggest that the ratio among the CBF/NF-Y subunits and c-Myc is important for the transcriptional activity of the wtB sequence, probably via the formation of either the CBF complexes targeting CCAAT or the c-Myc containing complex I on both HSP-MYC-B and CCAAT.

To further examine the involvement of complex I in the transcriptional activity of wtB and the regulation of the activity by c-Myc, bandshift assays were carried out using the nuclear extract prepared from cells transfected with the expression vector for c-Myc as above. Various amounts of c-myc expression vector, or the vector alone, were transfected to Balb 3T3 cells, and the nuclear extracts were prepared from transfected cells. The extracts were incubated with a labeled wtB probe and the nuclear extracts were prepared from transfected cells. The transfection of the vector alone did not affect the expression of c-Myc and CBF-C nor the electrophoretic patterns of complexes I and II (data not shown). In the cells transfected with the c-myc expression vector, c-Myc increased in a manner dependent on the dose of the c-myc expression vector (Fig. 7C). The formation of another DNA-protein complex I on the wtB probe was increased in cells transfected with the c-myc expression vector at low doses (5 or 10 ng) (Fig. 7C), where the transcriptional activity of wtB was enhanced (Fig. 7A). Complex I was then decreased in the cells transfected with the plasmid at high doses (50 or 100 ng), where the transcriptional activity was repressed (Fig. 7A and C). Complex II, on the other hand, was increased in a manner dependent on the dose of the c-myc expression vector (Fig. 7C). The formation of another DNA-protein complex, the Sp1 complex, was not affected by the transfection of the c-myc expression vector (Fig. 7D). These results indicate that the transcriptional enhancer activity of wtB is due to the formation of the specific complex I containing CBF proteins and c-Myc on the sequence and that c-Myc thus regulates the wtB enhancer activity via affecting different nucleoprotein complex formations on the wtB sequence: the balance of the amounts, as well as the binding affinity, of c-Myc, CBF proteins, and unidentified proteins involved in complexes I and II may control the transcriptional activity of wtB contributing to the hap70 gene expression.
were synchronized to the G0 phase by serum starvation, and cell cycle. To examine this possibility, mouse Balb 3T3 cells complex on the wtB sequence was thought to vary during the changes considerably during the cell cycle, the nucleoprotein labeled wtB probe and Western blot analyses (Fig. 8, cycle. The extracts were subjected to bandshift assays with a the nuclear extracts were prepared at various stages of the cell complexes formed on the wtB sequence is suggested to determine the G 1-specific transcription due to the sequence. A molecular model is shown.

Since the endogenous c-Myc expression gene expression is regulated by, at least to some extent, the formation of nucleoprotein complex I containing CBF proteins and c-Myc on both HSP-MYC-B and CCAAT in the wtB sequence and that the complex formation is controlled by the intracellular amounts of c-Myc.

We described the transcriptional regulation of hsp70 by c- myc through the formation of specific DNA-protein complexes. c-Myc associated with CBF/NF-Y to form a specific DNA-protein complex (complex I) on the wtB sequence in the hsp70 gene promoter containing the HSP-MYC-B element and the CCAAT box. c-Myc was also involved in another nucleoprotein complex (complex II) with unidentified proteins other than CBF/NF-Y on the same sequence. The in vivo ratio between complex I and complex II on the wtB sequence varied according to the cell cycle-dependent expression of c-myc expression. Complex I increased when the c-myc expression increased in the early G1 phase and then decreased when c-Myc was accumulated to reach a peak at the transition of G1 to S phase. Complex II, on the other hand, increased form the G1-S transition. The transcription due to the wtB sequence, as well as the transcription of the hsp70 gene, increased in the G1 phase to maximal expression at the same time of the maximal formation of complex I and then decreased with the decrease in the formation of complex I and the increase in the formation of complex II. c-Myc was thus thought to switch the up- and down-regulation of the hsp70 gene expression to show a peak in the G1 phase via transition between complex I and complex II on the wtB sequence.

c-Myc interacted with CBF-C directly and specifically in vitro. Unidentified factor(s) other than c-Myc and CBF were implied to be necessary for the formation of both complexes I and II, commonly or differently. Factor X, supposed to be involved at least complex I may directly bind to both c-Myc and the HSP-MYC-B element in the wtB sequence to yield complex I (Fig. 9). Factor X may also interact with CBF/NF-Y in complex I. Provided that factor X is a common factor to intermediate c-Myc and the HSP-MYC-B element, complex I containing CBF/NF-Y positively contributes to the transcriptional activity of the wtB sequence, while complex II without CBF/NF-Y is a transcriptionally inactive form. Alternatively, another factor,
factor Y, may be a bridge between c-Myc and the HSP-MYC-B element in complex II. The release of CBF/NF-Y from complex I to yield complex II, or the replacement of complex I containing factor X by complex II containing factor Y, was observed in cells transfected with a c-myc expression vector as well as in cells at the transition of the G2 to S phase of the cell cycle, where the expression level of c-Myc, but not of CBF, changed. During the S phase, the excess amount of c-Myc may upset the proper ratio between c-Myc and CBF/NF-Y to form complex I on the wtB sequence.

CBF/NF-Y is a well characterized transcription factor and expressed ubiquitously in a variety of mammalian cells. More than 170 genes have been found to contain the CBF/NF-Y-binding CCAAT sequence, and some of them were identified as target genes for CBF/NF-Y (43, 44). The human hsp70 gene contains two CCAAT sequences in the promoter region, at about −150 and −90 from the transcription start site (4). Both the CCAAT sequences were shown to be bound by NF-1/CTF in vitro (14), and furthermore, the CCAAT sequence around −90 was essential for the basal expression of the hsp70 gene (21). Another CBF of 114 kDa, different from CBF/NF-Y, was also reported to recognize the CCAAT sequence around −90 (13), and E1A and p53 competitively bind to the CBF of 114 kDa to stimulate or repress the hsp70 promoter activity, respectively (22, 23). In this report, we have shown that another CCAAT at −150 was recognized by CBF/NF-Y and that c-Myc makes complexes with CBF/NF-Y on the sequence to yield two opposite functions, i.e., activation and repression, on the hsp70 promoter. The up- and down-regulation by C-Myc via the sequence is thought to determine the cell cycle-dependent expression, namely, the maximal expression at the G1 phase, of the hsp70 gene. The two CCAAT sequences at −90 and −150 in the hsp70 promoter are thus thought to be involved in the basal and cell cycle-dependent expression of the gene, respectively.

The involvement of the CCAAT sequence in the cell cycle-dependent expression was also reported in the R2 gene of mouse ribonucleotide reductase (45) and in the cdc25c gene in which CBF/NF-Y interacted with Sp1 (46). Furthermore, CBF/NF-Y has recently been reported to interact with various proteins, including a TATA box-binding protein, TBP (37), histone HMG-I(Y) (49), and the human T-cell lympho-

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