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HSP-CBF Is an NF-Y-dependent Coactivator of the Heat Shock Promoters CCAAT Boxes*

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The cellular response to toxic stimuli is elicited through the expression of heat shock proteins, a transcriptional process that relies upon conserved DNA elements in the promoters: the Heat Shock Elements, activated by the heat shock factors, and the CCAAT boxes. The identity of the CCAAT activator(s) is unclear because two distinct entities, NF-Y and HSP-CBF, have been implicated in the HSP70 system. The former is a conserved ubiquitous trimer containing histone-like subunits, the latter a 110-kDa protein with an acidic N-terminal. We analyzed two CCAAT-containing promoters, HSP70 and HSP40, with recombinant NF-Y and HSP-CBF using electrophoretic mobility shift assay, protein-protein interactions, transfections and chromatin immunoprecipitation assays (ChIP) assays. Both recognize a common DNA-binding protein in nuclear extracts, identified *in vitro* and *in vivo* as NF-Y. Both CCAAT boxes show high affinity for recombinant NF-Y but not for HSP-CBF. However, HSP-CBF does activate HSP70 and HSP40 transcription under basal and heat shocked conditions; for doing so, it requires an intact NF-Y trimer as judged by cotransfections with a diagnostic NF-YA dominant negative vector. HSP-CBF interacts in solution and on DNA with the NF-Y trimer through an evolutionary conserved region. In yeast two-hybrid assays HSP-CBF interacts with NF-YB. These data implicate HSP-CBF as a non-DNA binding coactivator of heat shock genes that act on a DNA-bound NF-Y.

Protection from cellular stress is a fundamental function that enables all living organisms to counteract noxious environmental stimuli such as heat or toxic agents. A crucial aspect of the heat shock response is the rapid and massive production of distinct classes of related proteins conserved in evolution; based on their molecular weight, three major families of heat shock proteins (HSPs)[1] are catalogued: middle HSPs, 64/74 kDa HSPs; large HSPs, 90/110 kDa; and small HSPs, 27/40 kDa (1). The whole process is controlled by increasing the expression of the respective set of genes at the transcriptional level (2, 3). This phenomenon is mediated by the heat shock transcription factors (HSFs), which are present in a monomeric, non-DNA binding form in the cytoplasm of unstressed cells. Upon activation, HSFs rapidly form homotrimers, which translocate into the nucleus and bind to discrete palyndromic sites (heat shock elements (HSEs)) present in the promoters. In all cases tested so far, HSEs work in conjunction with, and indeed require, nearby elements such as GC and CCAAT boxes. Among heat shock promoters, HSP70s are perhaps the most thoroughly studied. In essentially all species HSP70 promoters contain CCAAT boxes (4–13). When multiple HSP70 genes are present in one species, such as in mouse and man, they all harbor one or multiple CCAAT sequences (4–6). Whenever tested in such diverse systems as mammalian cells, *Xenopus* oocytes, transgenic tobacco or zebrafish microinjection, the functional importance of CCAAT sequences has been clearly established (14–20). The exception is represented by *Drosophila*, which has no CCAAT boxes in their HSP70 promoters, a fact that is mirrored by the conspicuous absence of this element in any type of promoter. In addition to the HSP70 family, human HSP105 and HSP40 also contain CCAAT sequences, and as far as the HSP40 is concerned, they have also been shown to be important for expression (21, 22).

The CCAAT box is present not only in heat shock genes, but it is indeed one of the most widespread cis-acting elements, being found in 30% of eukaryotic promoters (23). In many DNA binding activators the acronym CCAAT is present: CTF/NF1 (CCAAT transcription factor), C/EBP (CCAAT/enhancer binding protein) and CDP (CCAAT displacement protein). However, in the HSP system two polypeptides have been directly implicated in CCAAT box function: NF-Y and CBF, hereafter renamed HSP-CBF to avoid confusion with CBF, which is another acronym of NF-Y (24, 25). NF-Y is a complex composed of three subunits: NF-YA, NF-YB, and NF-YC, all required for DNA binding. A large body of evidence indicates that NF-Y binds and activates many if not most CCAAT boxes in diverse promoters in different kingdoms: yeast, plants, and mammals (24, 25). Two of the subunits, NF-YB and NF-YC, contain histone-like domains required for dimerization, association of NF-YA and CCAAT binding. Two large Q-rich domains are present in NF-YA and NF-YC and function in activation assays. At first sight, the CCAAT boxes found in the HSP70 promoters across species match very well the NF-Y consensus obtained by the alignment of a compilation of >300 sites (26)[2]

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[1] The abbreviations used are: HSP, heat shock protein; HSF, heat shock transcription factor; HSE, heat shock element; wt, wild type; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; TnT, troponin T; ChIP, chromatin immunoprecipitation assay.

[2] R. Mantovani, unpublished data.

and experimentally derived with site-selection analysis (27). Analyzing the *Xenopus* HSP70, the group of A. Wollfe came to the conclusion that the CCAAT box is instrumental in maintaining an open chromatin configuration of the promoter so that HSF could rapidly activate (20). Evidence that *Xenopus* HSP70 activation is elicited through interactions of NF-Y with the p300 coactivator has been presented (28). *In vivo* footprinting experiments in mouse cells are supportive of this idea because CCAAT boxes are protected constitutively prior of the heat shock, whereas the HSEs become bound by HSFs activators only following the thermal stimulation (29, 30). In another report, NF-Y was shown to be the target of negative regulation by Myc to a −155 upstream site in the human HSP70 promoter (31), whose function is, however, unclear (15–17).

On the other hand, work on the human HSP70 promoter pointed to a different protein as crucial for CCAAT box activation. Screening of an expression library with multimerized HSP70 CCAAT-containing oligonucleotides lead to the identification of HSP-CBF, a cDNA coding for a 999-amino acid protein (32, 33). HSP-CBF is ubiquitous and highly conserved in many species; the *Saccharomyces cerevisiae* equivalent, Mak21p, is an essential gene (34). It contains a highly acidic N-terminal domain, usually a hallmark for transcriptional activators, and indeed expression of HSP-CBF in COS cells increased the activity of the human HSP70 promoter in a CCAAT-dependent way. GAL4 fusion experiments further pinpointed two regions important for transcriptional activation: the acidic N-terminal domain and a central part highly conserved across species (35). Interestingly, the N-terminal part of HSP-CBF is contacted by the viral oncogene E1A (36) and by the anti-oncogene p53 (37); E1A and p53 have opposite effects on HSP-CBF; the former increases, while the latter represses HSP-CBF-mediated activation (37, 38). To solve the issue of which protein is the *bona fide* activator of heat shock genes CCAAT boxes, we employed recombinant proteins in EMSA and immunoprecipitation assays *in vitro* and expression vectors and antibodies for chromatin immunoprecipitations *in vivo* on two promoters representative of the HSP70 and HSP40 families.

MATERIALS AND METHODS

Protein Production and Purification—HSP-CBF cDNAs (wt and mutants) were cloned into the His- and thioredoxin tagged PET32 expression vector using the *Nco*I site; mutants 1–533, 1–366, and 1–100 were derived by cutting with *Eco*RI, *Xho*I, and *Hin*dIII, respectively, and religating. Productions of the fusion proteins were as described in Refs. 39 and 40. The His-less NF-YB and NF-YA proteins were produced from PET3b, and NF-YC from the His-tagged PET32 was modified to eliminate the thioredoxin tag by cutting with *Nde*I and religating. NF-YA and NF-YB subunits were found in inclusion bodies and were resuspended in 6 M GnCl. Equimolar amounts of the recombinant proteins were renatured by slowly dialyzing away the denaturing agent, and the resulting material was centrifuged to remove precipitates, loaded on an NTA-agarose column, and purified according to standard procedures. HSP-CBF proteins were purified from soluble fractions. For production of HSP-CBF *in vitro*, the transcription/translation TnT system (Promega) was employed.

Immunoprecipitations—For protein-protein interaction studies, 50–100 ng of recombinant proteins were incubated in 200 μl of 300 mM KCl, 20 mM HEPES, pH 7.9, 0.05% Nonidet P-40, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, rotated for 2 h at 4 °C, and then added to 25 μl of protein G-Sepharose to which 5 μg of the anti-NF-YA Mab7 monoclonal antibody had been previously bound. Incubation was pursued for 2 h at 4 °C, unbound material was recovered after centrifugation, and the beads were washed with NDB100 with the addition of 0.1% Nonidet P-40. SDS buffer was added, and the samples were boiled at 90 °C for 5 min and loaded on SDS gels. Western blots were performed according to standard procedures with the indicated primary antibody and revealed with a Pierce peroxidase secondary antibody. The anti-HSP-CBF antibody was a kind gift of D. Linzer, Northwestern Univ., Evanston, IL.

EMSA—For electrophoretic mobility shift assays ^{32}P-labeled fragments, 10000 cpm, are incubated in NF-Y buffer (20 mM HEPES, pH 7.9, 50 mM NaCl, 5% glycerol, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol) with 5 μg of CH27 nuclear extract or with the recombinant proteins in a total volume of 10 μl; after incubation for 15 min at 20 °C, we added 2 μl of 1× NF-Y buffer containing bromphenol blue and samples loaded on a 4.5% polyacrylamide in 0.5× Tris borate EDTA. The oligonucleotides used were the following: HSP70, 5′-CTCATCGAGCTCGGTGATTGGCTCAGAAGGGAAAA-3′and 5′-TTTTCCCTTCTGAGCCAATCACCGAGCTCGATGAG-3′; and HSP40, 5′-AGGGCGGCGGCGATTGGCCGGCGCCGCGGG-3′ and 5′-CCCGCGGCGCCGGCCAATCGCCGCCGCCCT-3′. For supershift experiments, we used 300 ng of anti-NF-YA Mab7, anti-NF-YB, anti-NF-YC and anti-Gata1, and 100–300 ng of anti-HSP-CBF.

Transfections—The eukaryotic expression vector for HSP-CBF has been described (32). 2.5×10^4 COS or SAOS2 cells were transfected with 0.5 μl of LipofectAMINE (Life Technologies, Inc.) in a 24-well plate using different doses of the activating vectors, 0.1 μg of *Xenopus* HSP70-CAT (gift of N. Landsberger, Univ. Insubria, Varese, Italy) or HSP40-luciferase DNA (gift of K. Ohtsuka, Aichi Cancer Center, Nagoya, Japan), 50 ng of Nβ -galactopyranoside as an internal control and various amounts of carrier pGEM plasmid to keep the total DNA concentration constant at 1 μg. Cells were recovered 24/36 h after transfection, washed in phosphate-buffered solution (150 mM NaCl and 10 mM sodium phosphate, pH 7.4) and resuspended in TEN (150 mM NaCl and 40 mM Tris HCl, pH 7.4) for measurements of β-galactosidase and CAT or luciferase activities. Heat shocks were performed 30 h post-transfection, by placing the plates for 90 min at 42 °C; following a change of medium, cells were recovered after 5 h. A minimum of three independent transfections in duplicate were performed. Standard deviations represented <20% of the values.

Chromatin Immunoprecipitations—Formaldehyde cross-linking and chromatin immunoprecipitation was performed as described in Ref. 41. Exponentially growing HeLa cells were washed in phosphate-buffered solution and incubated for 10 min with the cross-linking solution, containing 1% formaldehyde; after quenching the reaction with glycine 0.1 M cross-linked material was broken by sonication into chromatin fragments of an average length of 500/1000 base pairs. Immunoprecipitation was performed with ProtG-Sepharose. The chromatin solution was precleared by adding ProtG-Sepharose for 15 min at 4 °C, aliquoted, and incubated with 2 μg of affinity-purified rabbit polyclonal antibodies for 3 h at 4 °C with mild shaking. Before use, ProtG-Sepharose was blocked with 1 μg/μl sheared herring sperm DNA and 1 μg/μl bovine serum albumin for 4 h at 4 °C and then incubated with chromatin and antibody overnight. Immunoprecipitates were eluted, and ethanol was precipitated. Recovered material was treated with proteinase K, extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated. The pellets were resuspended in 30 μl of H_2O and analyzed by using polymerase chain reaction with the following primers specific for the human HSP70 promoter: coding 5′-GGCGAAACCCCTGGAATATTCCCGA-3′, non-coding 5′-AGCCTTGGGACAACGGGAG-3′; for the DHFR promoter: coding 5′-GGCCTCGCCTGCACAAATAGGG-3′, non-coding 5′-GGGCAGAAATCAGCAACTGGGC-3′. The input sample was resuspended in 100 μl of H_2O and diluted 1:100.

Yeast Two-hybrid Assays—The yeast strain used was PJ69–4A, which contains three Gal4 inducible promoter elements fused to selection and reporter genes (42). This strain was transformed with plasmid pAS2–1 in which HSP-CBF-1–566 was cloned with *Nco*I-*Eco*RI in frame with the GAL4 DNA binding domain, AS2.1-HSP-CBFN, generating the strain PJ69-AS2.1-HSP-CBFN. This strain was then transformed with the pACT2 plasmid (CLONTECH) containing the NF-YB gene, cloned *Bgl*II-*Eco*RI in frame with the GAL4 activation domain. Transformants were plated onto synthetic media plates lacking histidine, adenine, tryptophan, and leucine and containing 2% dextrose as a carbon source and 1 mM 3-aminotriazole. His^+ colonies were assayed for β-galactosidase activity by a filter assay; colonies grown on selective plates were replica-plated on nitrocellulose filters, dipped in liquid nitrogen to permeabilize cells, thawed, and placed in a Petri dish containing Whatman No. 3MM paper saturated with Z buffer containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

RESULTS

NF-Y Not HSP-CBF Binds to HSP70 and HSP40 CCAAT Boxes with High Affinity—CCAAT boxes are conserved in the HSP70 promoters across species and are found, among others, in the human HSP40 promoter. To characterize the proteins binding to them, we performed EMSA analysis with nuclear extracts of mouse CH27 cells and two probes representative of

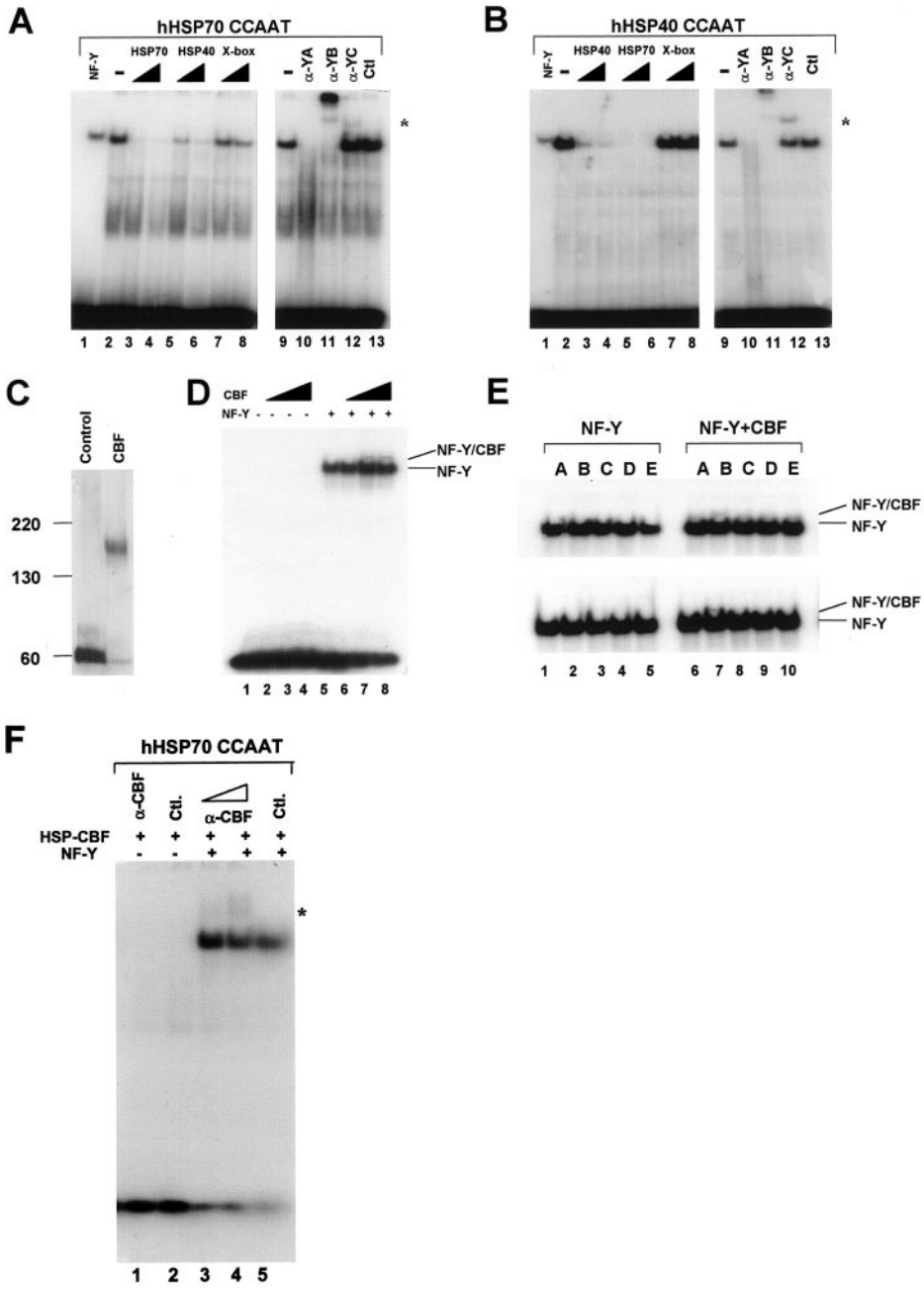

FIG. 1. **NF-Y is the HSP40 and HSP70 CCAAT-binding entity in nuclear extracts.** EMSA of NF-Y and HSP-CBF bound to the HSP70 (*A*) and HSP40 (*B*) proximal CCAAT box regions. *Lane 1*, 0.5 ng of recombinant NF-Y; *lanes 2–13*, CH27 nuclear extracts. *Lanes 2* and *9*, no competitor; *lanes 3* and *4* in *A* and *lanes 5* and *6* in *B*, 20- and 100-fold excess of cold competitor HSP70 oligonucleotide; *lanes 5* and *6* in *A* and *lanes 3* and *4* in *B*, 20- and 100-fold excess of cold competitor HSP40 oligonucleotide. *Lane 10*, anti-NF-YA Mab7, *lane 11*, anti NF-YB, *lane 12*, anti-NF-YC, *lane 13*, anti-Gata1. *C,* TnT expression of the control (Luciferase) and HSP-CBF labeled with [^{35}S]methionine. *D,* dose-response of *in vitro* translated HSP-CBF (0.5, 1, and 2 μl in *lanes 2–4* and *6–8*) without (*lanes 2–4*) and with 0.5 ng of recombinant NF-Y (*lanes 6–8*). *E,* NF-Y alone (*lanes 1–5*), or with 1 μl of HSP-CBF (*lanes 6–10*) on the HSP70 (*upper panels*) or HSP40 (*lower panels*) in different buffers. Buffer A: 50 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris HCl, pH 7.8, 0.5 mM EDTA, 5% glycerol. Buffer B: 50 mM NaCl, 10 mM Tris HCl, pH 7.8, 1 mM EDTA, 5% glycerol. Buffer C: 50 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris HCl, pH 7.8, 5% glycerol. Buffer D: 5 mM $MgCl_2$, 10 mM Tris HCl, pH 7.8, 0.5 mM EDTA, 5% glycerol. Buffer E: 1 mM $MgCl_2$, 10 mM Tris HCl, pH 7.8, 0.5 mM EDTA, 5% glycerol. *F,* antibody supershift of the NF-Y-HSP-CBF complex with anti-HSP-CBF antibodies on the HSP70 CCAAT box. *Lane 1,* HSP-CBF with 600 ng of purified anti-HSP-CBF purified antibody; *lane 2,* same with 600 ng of anti-Gata1 antibody; *lanes 3* and 4, 200 and 600 ng of anti-HSP-CBF antibody on NF-Y and HSP-CBF; *lane 5*, same as *lanes 3* and *4* except that the control anti-Gata1 antibody was used.

these families: the human HSP70 proximal −70 base pairs and human HSP40 proximal −100 base pairs CCAAT boxes, both shown to be essential for the full activities of the two promoters (15–17, 21). A single complex was observed with both oligonucleotides (Fig. 1, *A* and *B*, *lanes 2*). In parallel, we incubated *Escherichia coli*, produced NF-Y in the same assay, and promptly observed complexes (Fig. 1, *A* and *B*, *lanes 1*); it was immediately apparent that the complexes generated with nuclear extracts and with recombinant NF-Y had similar electrophoretic mobilities. Both the HSP70 and HSP40 complexes were specifically self- and cross-competed by unlabeled oligonucleotides containing the two CCAAT boxes but not by an unrelated oligonucleotide harboring the MHC class II Ea X box (Fig. 1, *A* and *B*, *lanes 3–8*). These complexes were also supershifted by anti-NF-YB and anti-NF-YC antibodies and inhibited by the anti-NF-YA Mab7 monoclonal (Fig. 1, *A* and *B*, *lanes 9–13*). These results establish conclusively that the more readably seen DNA-binding protein in EMSA assays is NF-Y and represents the first demonstration that NF-Y is the HSP40 proximal CCAAT-binding protein.

In an initial attempt to verify the relative affinity of NF-Y and HSP-CBF for the CCAAT boxes of the HSP genes, we cloned HSP-CBF into the PET32 *E. coli* expression vector. Recombinant HSP-CBF was produced and purified using the His-tag of the protein on NTA nickel columns; most of the resulting HSP-CBF is proteolytically cut to a size of 55 kDa. We employed this material in EMSA analysis; no binding of the recombinant HSP-CBF was observed on either targets even at very high dosages (1 μg, not shown). Because of the poor yield of intact bacterial HSP-CBF, we turned to a TnT transcription/translation system; HSP-CBF was abundantly produced in an intact form as checked by [^{35}S]methionine labeling of the protein (Fig. 1*C*). This material was used for EMSA as above, and again no binding was observed. We systematically modified the concentrations of salts, divalent cations, and detergents; we were incapable of observing specific CCAAT binding of HSP-CBF on the HSP70 or HSP40 CCAAT probes (Fig. 1*D*, *lanes 1–4*). EMSAs with HSP70 and HSP40 CCAAT oligonucleotides

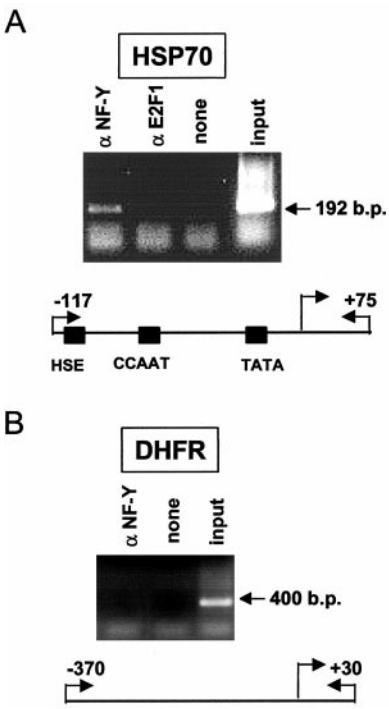

FIG. 2. **NF-Y binds the HSP70 promoter *in vivo*.** Chromatin immunoprecipitation analysis of NF-Y binding to HSP70 CCAAT box region *in vivo*. From the same HeLa immunoprecipitates corresponding to the anti-NF-YB and anti-E2F1 (Santa Cruz) antibodies, DNA corresponding to the HSP70 (*A*) and DHFR (*B*) were polymerase chain reaction amplified with the indicated oligonucleotides.

were then performed with both recombinant proteins. Addition of the two proteins in the buffer conditions described in Ref. 32 yielded a faint complex migrating more slowly than the prevalent NF-Y complex (Fig. 1*D*, *lanes 5–8*); this upper complex was NF-Y-dependent because it was competed by cold CCAAT oligonucleotides and supershifted by anti-NF-Y antibodies (not shown). In no other buffer conditions was this upper complex detectable with the HSP40 or the HSP70 probe (Fig. 1*E*). The presence of HSP-CBF in the higher complexes was confirmed by supershift experiments with purified rabbit antibodies against HSP-CBF (Fig. 1*F*). Although these data do not rule out completely the possibility that HSP-CBF binds DNA, they establish (i) that a clear difference in affinities between recombinant NF-Y and HSP-CBF exists under standard EMSA conditions, and (ii) that NF-Y can associate HSP-CBF, albeit with apparent modest affinity and strict buffer requirements, when bound to DNA *in vitro*.

Interactions of NF-Y with the HSP70 CCAAT Box in Vivo—To verify the ability of NF-Y to interact with the HSP70 promoter *in vivo,* we employed ChIP on growing HeLa cells. The cross-linked chromatin was immunoprecipitated using a polyclonal antibody against NF-YB. As negative controls, we included a reaction lacking a primary antibody and one that contained an antibody against E2F1, an abundant nuclear transcription factor that does not have a binding site on the HSP70 promoter. After immunoprecipitation and reversal of the cross-links, enrichment of the endogenous HSP70 promoter fragment in each sample was monitored by polymerase chain reaction amplification using primers amplifying the human HSP70 promoter region from −117 to +75 base pairs. The results show that only the anti-NF-Y antibody immunoprecipitates chromatin containing the HSP70 promoter (Fig. 2*A*). This result mirrors the binding of NF-Y to other promoters of cell cycle-regulated genes[3]. To rule out unspecific effects of the ChIP assays performed with the anti-NF-YB antibody, the same immunoprecipitate was also used to amplify DHFR, a CCAAT-less cell cycle-regulated promoter; no amplification was observed (Fig. 2*B*), which is consistent with the lack of NF-Y binding sites on this promoter. These results provide *in vivo* evidence for a specific retention of NF-Y to the HSP70 promoter, indicating that this transcription factor plays a critical role in the activation of the HSP70 gene.

HSP-CBF Activates HSP70 and HSP40 in an NF-Y-dependent Manner—Functional assays have indicated that the human HSP70 promoter is activated in a CCAAT-dependent way by HSP-CBF (32, 35). Given the results obtained in our *in vitro* EMSA analysis, we felt it important to verify this point. To this aim, we cotransfected a CAT reporter gene driven by the *Xenopus* HSP70 promoter that shows identical architecture and similar sequence to the human counterpart with a HSP-CBF expression plasmid in COS and SAOS2 cells. COS cells were chosen because, unlike CHO cells, they were shown to be permissive to HSP-CBF activity; the $p53^{-/-}$ SAOS2 were chosen because of the possible interfering role of p53 on HSP-CBF activation (37). As shown in Fig. 3*A*, HSP-CBF overexpression leads to a 4–5-fold increase in promoter activity in COS cells. The effect is strictly dependent upon the integrity of the CCAAT boxes because a promoter containing mutations in the two CCAAT boxes, HSP70mut in Fig. 3*A,* does not respond to HSP-CBF overexpression. To establish the specific role of NF-Y in such system, we cotransfected a highly diagnostic vector coding for the DNA binding-defective NF-YAm29 mutant; the protein produced by this construct acts in a dominant negative fashion associating the NF-YB-NF-YC dimer and preventing the endogenous trimer to bind CCAAT boxes (43). Under these conditions, CAT activity was not enhanced by HSP-CBF. Next, we heat shocked COS cells cotransfected as above and found that (i) the levels of CAT activity are, as expected, increased upon heat treatment, an effect that requires intact CCAAT boxes (20, 44); (ii) HSP-CBF further increased overall activity; and (iii) cotransfections with the dominant negative vector abolished the positive effect of the heat shock and of HSP-CBF overexpression (Fig. 3*B*).

In another set of conceptually similar experiments, we employed the human HSP40 promoter, from position −227 to +30, which contains two CCAAT boxes at −100 and −220 and an HSE at -70. This promoter is inducible upon heat shock at 42 °C in HeLa cells (21). Again, we observed a robust effect of HSP-CBF, both under basal and after heat treatment, and a negative effect of cotransfecting the dominant negative vector (Fig. 3*B*). The same experiments were performed in SAOS2 cells, and we obtained equivalent results on both promoters (data not shown). From this set of experiments, we conclude that (i) HSP-CBF is an activator of HSP transcription beyond the HSP70 system and (ii) that the integrity of the CCAAT box and a functional DNA binding NF-Y trimer are required.

HSP-CBF Is Not a General Coactivator—The results shown above raised questions about the specificity of the transcriptional activity of HSP-CBF for NF-Y and/or for heat shock promoters. To verify this, we used other promoters in the same cotransfection systems. HSP70 genes are regulated during the cell cycle, being activated at the G_1/S boundary (45, 46), thus it could be conceivable that HSP-CBF activity might be involved in the large family of CCAAT-containing cell cycle-regulated promoters (26). We employed several cell cycle-regulated promoters either containing *bona fide* NF-Y binding sites, such as TK, or lacking CCAAT boxes, such as DHFR and Cyclin E. As

[3] A. Gurtner and G. Piaggio, submitted for publication.

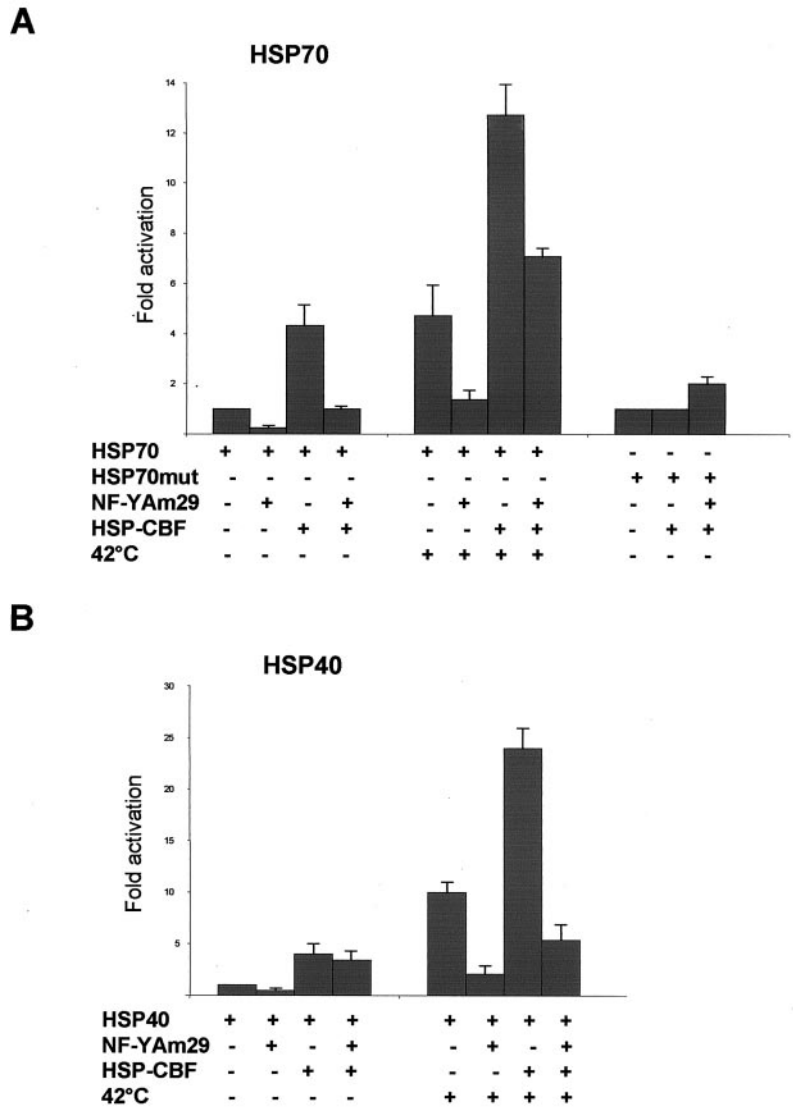

FIG. 3. **HSP-CBF transactivation of HSP70 and HSP40 promoters requires NF-Y.** Transfections of COS cells *A, Xenopus* wt or CCAAT-mutated HSP70 promoters fused to the CAT reporter gene (250 ng) were assayed with 300 ng of HSP-CBF, with or without 100 ng of NF-YAm29 dominant negative vector under basal or heat shocked conditions. *B,* same as *A* except that 200 ng of HSP40 Luciferase gene (21) was used. The data refer to the average fold induction (similar data were obtained in SAOS2 cells). Standard deviations are indicated by *error bars*.

a further control, we used the tissue-specific MHC class II Ea that is highly dependent upon NF-Y binding. Cotransfections of increasing doses of HSP-CBF with all these reporters yielded levels of transcriptional activities that were not augmented and, in fact to various extents, decreased when compared with transfections in the absence of HSP-CBF (Fig. 4). These results rule out that HSP-CBF is a general cofactor activating all types of promoters and further support the idea that not all CCAAT-boxes are activated because TK and Ea are clearly unaffected.

NF-Y and HSP-CBF Binds in Vitro and in Vivo—A possible explanation for the experiments shown above is that NF-Y and HSP-CBF can bind to each other, and thus NF-Y could recruit HSP-CBF onto the promoter. We started to evaluate this possibility by performing immunoprecipitations with the recombinant proteins and the anti-NF-YA monoclonal antibody Mab7. Using the wt HSP-CBF and NF-Y trimer, we observed specific interactions between the two proteins because Western blots revealed HSP-CBF in the bound fraction of the material immunoprecipitated with Mab7 but not with an irrelevant anti-Gata1 antibody (Fig. 5, *upper panel*). As expected, NF-Y subunits were also found in the same bound fractions as exemplified for NF-YB (Fig. 5 and data not shown). Deletion mutants of HSP-CBF were produced and assayed; HSP-CBF-1–533 and HSP-CBF-1–366 were still capable to bind NF-Y, although HSP-CBF-1–100 was not. We then checked the protein-protein interactions of HSP-CBF-1–533 separately with

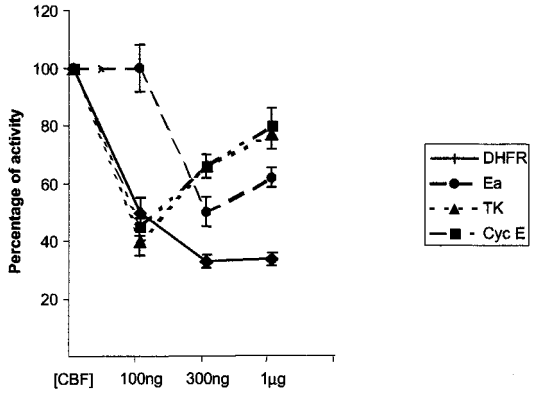

FIG. 4. **HSP-CBF is not a general coactivator.** The DHFR, Ea, TK, and CyclinE reporters were transiently transfected in COS cells alone or together with 100 ng, 300 ng, 1 μg of HSP-CBF. The average activities are shown and standard deviations were <20%.

NF-YA and with the NF-YB-NF-YC dimer; as shown in the *lower panel* of Fig. 5, HSP-CBF is incapable of associating the separate NF-Y subunits. We conclude that the NF-Y trimer, but not the single subunits, is able to interact with a domain of HSP-CBF located between amino acids 100 and 366.

To verify the interactions in an *in vivo* system, we employed the two hybrid assay in yeast. We cloned the HSP-CBF-1–533

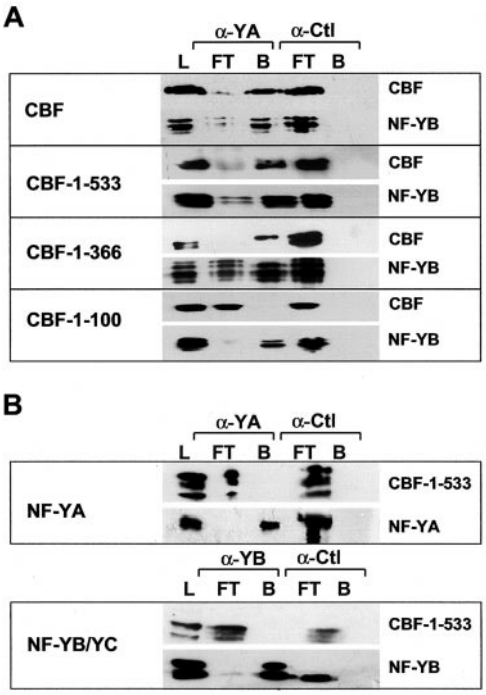

FIG. 5. **NF-Y-HSP-CBF protein-protein interactions *in vitro*.** Immunoprecipitations of recombinant proteins with the anti-NF-YA Mab7 monoclonal antibody or with a control anti-Gata1 are shown. *L*, load material; *FT*, follow-through, unbound proteins; *B*, bound material. *A,* recombinant NF-Y trimer and the wt or the indicated HSP-CBF mutants were used. Immunoblots were performed with anti-HSP-CBF and anti-NF-YB antibodies. *B,* we used the separated NF-YA (*upper panel*) or NF-YB-NF-YC dimer (*lower panel*) with Mab7 and anti-NF-YB antibodies, respectively.

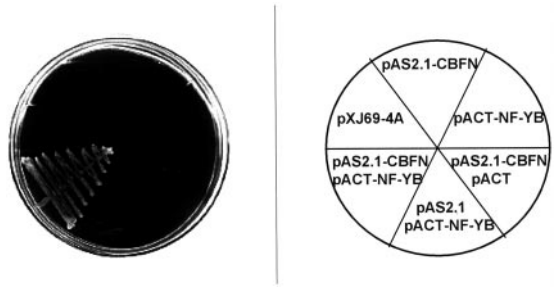

FIG. 6. **NF-YB interacts with HSP-CBF in yeast.** Yeast strain PJ69–4A was transformed with the indicated plasmids and grown in a Ade-Leu-Trp-His- minimal plate in the presence of 1 mM 3-AT.

mutant that interacts with NF-Y *in vitro* into the pAS2.1 vector containing the DNA-binding domain of GAL4 deriving pAS2.1-CBFN and NF-YB in the pACT vector, in frame with the GAL4 activation domain; these constructs were introduced in the PJ69–4A yeast strain and selected for Leu and Trp auxotrophy. This strain contains Ade and His selectable markers and a LacZ reporter gene that are under the control of three different GAL4-dependent promoters, Gal2, Gal1, and Gal7, respectively (42). On Leu^- Trp^- Ade^- His^- plates, robust growth was observed of the double transformants containing pACT-NF-YB and pAS2.1-CBFN but not of the transformants containing either plasmids with the empty counterpart or of the single plasmids (Fig. 6). The resulting colonies are white, an indication of adenine auxotrophy, and positive in β-galactopyranoside assays (data not shown). This proves that a functional interaction occurs between NF-YB and HSP-CBF. It should be noted that the yeast *S. cerevisiae* does contain NF-Y homologs, the HAP2/3/5 complex involved in activation of cytochrome genes (Ref. 47 and references therein), and that the yeast subunits can associate the mammalian homologs (48). Thus we cannot exclude the possibility that the overexpressed mouse NF-YB is in reality complexed with the yeast HAP2/5 and present as a trimeric complex; in this case HSP-CBF would interact with a hybrid trimer in yeast. Overall, these data are in agreement with the *in vitro* immunoprecipitations, and we can conclude that HSP-CBF and NF-Y can form complexes in solution both *in vitro* and *in vivo*.

DISCUSSION

The CCAAT-boxes are extremely conserved in the promoters of heat shock genes; in the HSP70 family, they have been found in mammals, chicken, *Xenopus*, algae, oomycetes, fish, and parasites (4–14). In this report, we clarified an important point for our understanding of the molecular mechanisms leading to the induction of these genes, namely the identity of the proteins that activate such sequence. Our data obtained with EMSA *in vitro*, with ChIP assays *in vivo,* and with transfections with an NF-YA dominant negative vector conclusively establish NF-Y as the DNA-binding protein that recognizes HSP CCAAT boxes. However, our transfection results revealed that HSP-CBF is a gene-specific coactivator of heat shock promoters, at least of those, the vast majority, containing this element.

It has long been known that the CCAAT boxes play a crucial role in HSP transcription. Previous work performed in *Xenopus* oocytes on the HSP70 promoter indicated that they are essential for induction after heat treatment. Most importantly, Wolffe's laboratory found that their elimination brought profound alterations to the "open" chromatin configuration that normally allows HSFs to bind and activate transcription shortly after the stimulus. Indeed in CCAAT-less constructs the whole promoter remains in a tight, closed nucleosomal configuration, inaccessible to HSFs and to the factors binding to the core promoter sequences (20). In a later study, the same authors investigated the NF-YB relationship with the coactivator p300, which possesses a histone acetyltransferase activity important for transcriptional activation. p300 acetylates the NF-YB subunit of NF-Y, but the function of this modification, if any, remained unclear (30). Formal proof that NF-Y was the protein involved *in vivo* was, however, was lacking. These results lead to the hypothesis that NF-Y presets chromatin configuration for activators to bind nearby and for coactivators to be recruited, which was in full agreement with our own data on the MHC class II Ea promoter performed with nucleosomal and chromatin reconstitution systems *in vitro*. We found that NF-Y has a high intrinsic affinity for nucleosomal structures thanks to the NF-YB-NF-YC histone-like subunits (39, 40). Consistent with this picture, previous *in vivo* footprinting studies in mammalian cells showed that the CCAAT box of the HSP70 promoter was protected constitutively even in unshocked cells (29, 30).

However, in the human system a different protein was shown to activate the CCAAT-sequence; HSP-CBF, isolated thanks to library screenings with multimerized human HSP70 CCAAT boxes, showed typical features of activators and indeed behaved so (32). Although clear-cut evidence of specific CCAAT binding activity by HSP-CBF was circumstantial, additional features of HSP-CBF matched quite well the overall regulation of HSP70 promoters; binding to the CR3 region of the 13SE1A, a positive coactivator of heat shock genes (38), correlated with strong superactivation of GAL4-HSP-CBF fusions (36). On the other hand, binding to p53, a repressor of HSP transcription, lead to inhibition of HSP-CBF activity (37). The crucial role of HSP-CBF in cellular functions is further supported by the following observations: (i) HSP-CBF genes can be retrieved from expressed sequence tags and genomic data banks of a number of species including zebrafish, *Caenorhabditis elegans*,

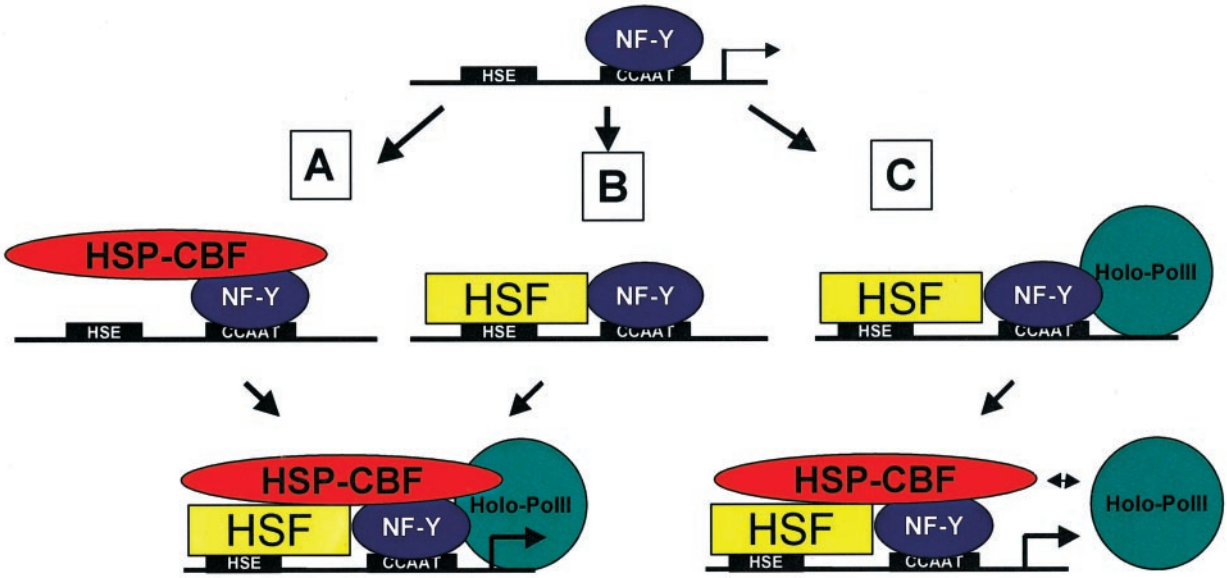

FIG. 7. **Possible mechanisms of HSP-CBF-mediated activation.**

Drosophila melanogaster, *S. cerevisiae,* and *Schizosaccharomyces pombe* in addition to mouse, rat, and man. (ii) Mak21p, the yeast homolog of HSP-CBF, was isolated in a screening to complement mutations in genes allowing propagation of M1 double-stranded RNA and was shown to be essential for cell growth, apparently being required for 60 S ribosomal biogenesis. The lack of complementation of the Mak21–1 phenotype by the human gene, suggesting notable interspecies differences and the absence of yeast conditional mutants limit inferences from the yeast studies.

We were unable to detect any sequence-specific DNA binding capacity of HSP-CBF on CCAAT oligonucleotides. On the other hand, clear-cut results *in vitro* and *in vivo* implicate NF-Y as the DNA binding factor. It is unclear to us how the original screenings yielded such polypeptide, but the likeliest explanation might be that HSP-CBF has affinity for multimerized CCAAT boxes or for random longer pieces of DNA in general irrespective of the sequence. Preliminary evidence of this has been obtained in our laboratory.[4] These discrepancies notwithstanding, it is clear that our data are entirely consistent with the previous functional indications, but point to a fundamentally different role of HSP-CBF in HSP activation, not direct sequence-specific DNA binding but association with a CCAAT-bound NF-Y. Our protein-protein interaction analysis identified the region of HSP-CBF-NF-Y interactions between amino acids 100 and 366 of HSP-CBF. Interestingly, this conserved region partially overlaps with the acidic N-terminal region contacted by E1A and p53 (36, 37) and is distinct from another domain, also evolutionarily conserved, that is important for HSP transactivation (35). It is tempting to speculate that HSP-CBF could be a protein scaffold that integrates interactions with different transcription factors, both upstream and in the core promoter.

What would be the functional relationships between NF-Y and the HSP-CBF coactivator? Coactivators are a large and heterogenous family of molecules that do not bind DNA directly but act on prebound sequence-specific factors. Many coactivators, such as CBP/p300, PCAF, GCN5, and PC4 (reviewed in Ref. 49) are considered as general cofactors that act on many if not most genes. Another large family of coactivators are factor-specific, that is, they recognize specific classes of DNA binding activators. Upon hormonal induction, for example, nuclear receptors bind coactivators that displace corepressor molecules. Interestingly, many of these factor-specific coactivators possess intrinsic HAT activities, to some extent similar to those of p300/CBP (50). Our data on the cell cycle promoters, as well as previous findings on the α-globin, clearly rule out that HSP-CBF is an NF-Y-specific coactivator because many NF-Y-dependent promoters are not responsive to HSP-CBF. A third class of coactivators is gene-specific; a prototypical example is represented by CIITA, the master switch regulator of genes involved in antigen presentation (51). CIITA does not bind DNA directly, but it recognizes a nucleoprotein platform composed of RFX, CREB, and NF-Y that binds to the conserved S-X-X2-Y boxes in all genes concerned. Upon overexpression in cells, it has some affinity for the single factors, NF-Y and RFX, but it is incapable of associating either factor in DNA-binding assays. It contains a highly acidic N-terminal part that is essential for transcriptional activation. All these features are highly reminiscent of the HSP-CBF activities described here and in previous studies.

Our model predicting that HSP-CBF is a gene-specific coactivator brings an important corollary: HSP-CBF makes contacts with NF-Y and, consistent with this, it activates basal transcription provided that an intact CCAAT box is present. However, it also activates heat shocked transcription under conditions in which it is conceivable that it could make important connections with HSFs. The apparent weak affinity of a CCAAT-bound NF-Y for HSP-CBF, as detected in the experiments shown in Fig. 1, suggests that additional factors, such as HSFs, and longer fragments of DNA are required for stable interactions. GC boxes, presumably binding to members of the Sp1 family, are also believed to play a role, at least in human HSP70, and could be important in the recruitment mechanism.

The mechanistic details of activation cannot be distinguished by our experiments, but three possibilities can be envisaged. (i) HSP-CBF might intervene early during the establishment of an open chromatin configuration within the heat shock regulatory regions following NF-Y binding (Fig. 7*A*). (ii) Alternatively, it could be recruited only when HSFs binds to the HSEs and increase the stability of the nucleoprotein complex (Fig. 7*B*). The highly acidic domain, which is required but not sufficient for activation, would make contacts with proteins of the basal machinery, such as some of the TAF_{II}s. In these two

[4] C. Imbriano and R. Mantovai, unpublished data.

schemes HSP-CBF might be important for chromatin remodeling and would therefore play a role similar to p300/CBP. (iii) It could help the release of the stalled polymerase by weakening the contacts between the basal factors and the upstream activators (Fig. 7*C*, see Ref. 52). In *Drosophila*, the elongation factor P-TEFb is required for productive transcriptional elongation of HSP70 mRNA, and HSF is required but not sufficient for P-TEFb recruitment (53). In this scenario, HSP-CBF might play a role in the post-HSF recruitment phase of transcriptional activation. It is worth reminding that the CIITA coactivator has been shown to be contacting DNA-bound activators, proteins of the basal machinery, as well as the elongation factor P-TEFb (51).

Finally, results presented here have wider implications that potentially apply to other systems; HSPs, in fact, are but one category of genes that are up-regulated by an increase in temperature. MDR1 and Erp72/Grp-78 families are also activated, but in a HSE-independent manner. Grp78 is evolutionarily related to HSPs and belongs to the chaperone endoplasmic reticulum proteins involved in the unfolded protein response (54–56). Genes coding for such proteins, which include Grp94, calreticulin, protein disulfide isomerase, and Herp are activated through a conserved composite promoter sequence termed ERSE (54–60). Molecular dissection of ERSEs have detailed that they contain a CCAAT box, and indeed heavily rely on NF-Y for their function (52–54), and also contain an ATF6 binding sequence spaced by a linker of variable sequence and conserved distance (58, 60). It has been proposed that ATF6, a DNA-binding transcription factor normally residing in the endoplasmic reticulum, is cleaved upon endoplasmic reticulum stress; the resulting product unmasks a hidden activation domain that upon binding of ATF6 close to NF-Y strongly activates transcription. Cooperative binding between the two activators on DNA is essential (58). An intriguing possibility is that HSP-CBF might serve as a more general coactivator of a cellular "stress response" by functioning also on the ATF6-NF-Y couple. The reagents and assays developed here will help elucidate these points.

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