Maximal Stress-Induced Transcription from the Human *HSP70* Promoter Requires Interactions with the Basal Promoter Elements Independent of Rotational Alignment

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Transcription of the human *HSP70* gene is regulated by a complex array of *cis*-acting promoter elements that respond to conditions that include normal conditions of cell growth and induction following physiological stress. We have examined the requirements of the basal and inducible promoter elements by using promoter mutations and a transient transfection assay. Multiple forms of stress-induced transcription, including heat shock and incubation with heavy metals or amino acid analogs, are mediated by a single heat shock element (HSE) between −105 and −91 consisting of three contiguous 5-base-pair units, NGAAN, that are inverted relative to adjacent units. Maximal inducible expression requires a fully functional basal promoter. Spacing mutations which alter the relative helical orientation of adjacent genetic elements have only minimal effects on basal and stress-inducible expression and show no effects of periodicity. In addition, placement of the HSE adjacent to the basal promoter removes the requirements for a fully functional basal promoter for maximal stress-inducible expression. These results suggest that factors bound at the HSE and the basal promoter can function through multiple interactions.

The transcriptional regulation of heat shock genes has provided a model system for the study of eucaryotic gene expression (19). Sequence comparisons of *Drosophila* heat shock promoters revealed a conserved 14-base-pair (bp) inverted repeat, C--GAA--TTC--G (14, 31), designated as the heat shock element (HSE), which has subsequently been found as multiple arrays upstream of every heat shock gene (5, 30, 38). The position of the most proximal HSE in *Drosophila* heat shock genes is typically 15 to 18 bp upstream of the TATA box; however, variable distances between these elements are typical for vertebrate heat shock genes (5).

The analysis of the chicken (26), *Xenopus* (3, 4), mouse (47), and human (40, 43) *HSP70* promoters has revealed additional genetic elements, such as GC, CCAAT, activating transcription factor (ATF), and AP-2 motifs which participate in modes of regulation other than heat induction (3, 4, 40, 43). The human *HSP70* gene has a basal level of expression which is induced by multiple forms of stress, including heat shock and incubation with heavy metal ions or amino acid analogs. In addition, *HSP70* gene expression is cell cycle dependent (23) and serum responsive (44). The actions of several oncogenes, including the adenovirus E1a 13S protein (29, 42), c-*fos* (34), c-*myc* (15), and T antigen (17), have been shown to modulate *HSP70* transcription. Transfected *HSP70* promoter constructs are forskolin responsive in PC-12 cells (G. T. Williams, unpublished observations) and hemin inducible during erythroid differentiation in K562 cells (39).

Human *HSP70* basal transcription is mediated by a compact multicomponent array of promoter elements that ex-

tends to −74 relative to the start of transcription (12, 40, 43). Transfection experiments with 5′ deletions and linker scanner (LS) constructs have demonstrated that basal expression represents the contributions of multiple elements, including the CCAAT box at −67, the GC box at −48, the TATA box at −28, and an ATF- or AP1-like element at −37 (12, 40, 43) (see Fig. 1). Several regulatory pathways, including those induced by serum (43), adenovirus E1a (40), or forskolin (Williams, unpublished observations), are known to be mediated through these basal elements. These studies have been complemented by in vitro transcription factor-DNA-binding experiments which have characterized the interactions of CCAAT transcription factor (CTF [25]) Sp1 (24), TFIID (28), and AP2 (W. Morgan, unpublished observations) with the basal promoter sequences. A summary of these in vitro studies is depicted in Fig. 1.

The sequences required for heat shock or metal responsiveness map to a distal domain between −107 and −68 (43) (Fig. 1). Within this domain is an HSE centered at −100 which binds to the heat shock factor (HSF) (18, 25, 27). Distinct HSE-binding activities have been detected in extracts from control and stressed HeLa cells (27). The appearance of stress-induced HSE-binding activity, whether resulting from heat shock, metal ion, or amino acid analog treatment, parallels the kinetics of *HSP70* gene transcription (27). The precise details of the mechanism of HSF activation and the nature of the interaction between activated HSF and the general transcriptional machinery remain unclear.

In this paper we examine the interactions between the factors bound to the promoter region conferring stress inducibility and those bound to the promoter region mediating basal expression. By analyzing a collection of mutated *HSP70* promoter constructs, we demonstrate that a single HSE is responsible for heat shock-, heavy metal-, and amino acid analog-induced transcription. Although none of the

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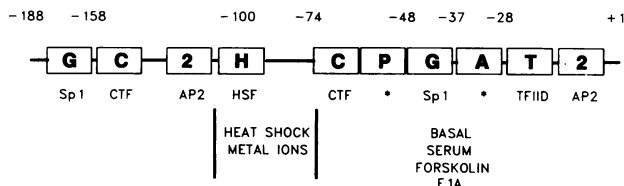† Present address: Department of Genetics, University of Illinois at Chicago, Chicago, IL 60612.

FIG. 1. Schematic of human *HSP70* promoter. Promoter sequences from −1 to −188 are represented schematically by a line. The boxes represent the approximate locations of transcription factor-binding sites. The factors that have been shown to bind to these regions are indicated below the boxes (CCAAT transcription factor [CTF] and HSF). Sites where binding activity has been detected but not identified are marked with an asterisk (*). The corresponding genetic elements located in these regions are indicated in the boxes (GC element [G], CCAAT element [C], AP2 element [2], heat shock element [H], purine-rich sequences [P], ATF- or AP1-like element [A], and TATA element [T]). The protein-DNA interactions were characterized by DNase I footprinting (G, C, 2, H, T), by exonuclease III footprinting (P) (Wu et al. [45]) and by gel mobility shift assays (C, H, A). A summary of 5′ deletion transfection experiments is shown below the factors. Various forms of regulation are indicated below the promoter regions which confer them.

mutations in the basal promoter abolish stress inducibility, mutations that reduce basal expression also reduce the absolute levels of stress-induced expression. Spacing mutations (SM) which periodically vary the relative spacing and rotational alignment between different regions of the promoter have minimal effects on inducible expression, thus indicating a surprising flexibility in the mechanism of heat shock promoter transcriptional regulation.

MATERIALS AND METHODS

Plasmid constructions. The details of the promoter mutagenesis strategy and the construction of the transfection vector (Fig. 2) containing the chloramphenicol acetyltransferase (*CAT*) test gene and the neomycin phosphotransferase gene (*NEO*) regulated by 74 bp of the human *HSP70* promoter have been described previously (40). The transfection vector was constructed such that various promoter mutations could be shuttled into a common vector background containing the covalently linked internal control *HSP-NEO* gene. LS mutations, SM, or internal deletions (ID), each in the context of a −188 promoter, and 5′ deletions (Δ5) or LS mutations in the context of a −100 promoter (LSP) were inserted into the promoter cassette of the transfection vector upstream of the *CAT* gene by using the unique *NdeI* and *MstII* restriction sites, generating the LSN, SMN, Δ5N, or LSPN series, respectively. Thus, when a construct designation includes an N, this signifies that the given promoter mutation is present in the transfection vector background.

The nomenclature for each of the constructs in the LSN, SMN, and IDN series refers to the parental 3′ and 5′ deletions that were used to generate the given mutation. In addition, the spacing change for each SM construct is indicated after a plus sign. Fusion of the *BglII* sites at the endpoints of each deletion places 10 bp of linker sequence between the given *HSP70* promoter fragments. LSN −107/−100 is the only exception to this construction strategy. For its construction, a 5′ deletion containing wild-type (WT) sequences to −100 (no *BglII* linker), generated by *SspI* digestion, was recombined by blunt-end ligation with a 3′ deletion, Δ3/−107, whose *BglII* site had been filled in by the
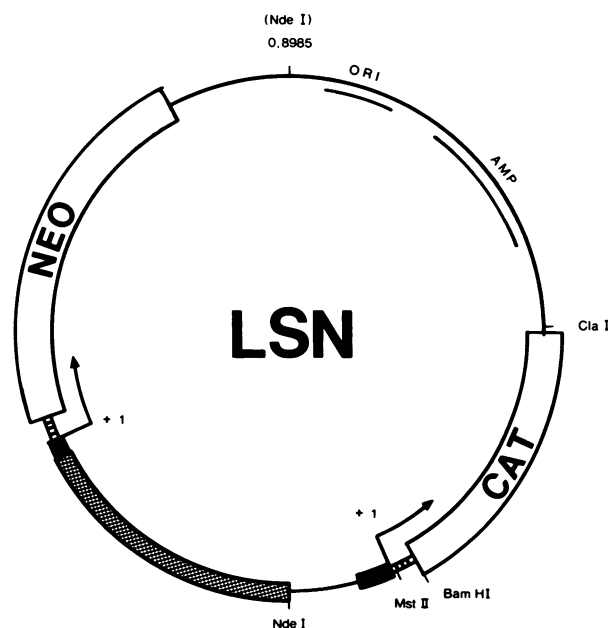

FIG. 2. Features of transfection vector. The transfection vector contains (i) a promoter cassette into which all of our promoter mutations can be shuttled on an *NdeI-MstII* restriction fragment (the *NdeI* site in parentheses has been destroyed), (ii) a test gene cassette containing the *CAT* gene, and (iii) a covalently linked *NEO* gene fused to 74 bp of the human *HSP70* promoter which can serve both as an internal control and a selectable marker. The schematic is denoted LSN, which is indicative of a construct containing an LS mutation (−188) in its promoter cassette. Replacement of the cassette with a Δ5, ID, SM, or LSP generates a Δ5N, IDN, SMN, or LSPN construct, respectively. ■■, Human *HSP70* promoter sequences; ▥▥, human *HSP70* untranslated sequences; ▢, bacterial genes; ──, vector sequences; ▨, λ spacer DNA.

Klenow fragment of DNA polymerase I (Klenow). In some cases, LSN −69/−55, LSN −40/−26, SMN −40/−42 +16, SMN −37/−42 +19, SMN −61/−63 +16, SMN −57/−63 +20, SMN −73/−74 +15, SMN −69/−74 +19, and the *BglII* site has been filled in and religated, resulting in 14 bp of linker sequence.

Δ5N/−105 is a 5′ deletion mutation containing WT sequences to −105 (40). LSPN WT is similar to LSN WT except that the sequences between −100 and −188 have been removed, as described by Williams et al. (40). LSPN WT* is similar to LSPN WT except that the vector sequences which flank the HSE in Δ5N/−105 have been inserted at the corresponding location in LSPN WT. Δ5/−105 was digested with *BglII*, the ends were filled in Klenow, and an *SphI* site was created by using linkers. After isolating the *NdeI-SphI* fragment from this construct, the new *SphI* site was fused to the *SphI* site (−100) in LSP WT, replacing the corresponding *NdeI-SphI* fragment. The *NdeI-MstII* promoter-containing fragment from the resulting construct, LSP WT*, was shuttled into the transfection vector, generating LSPN WT*.

Although LSN −44/−35 and LSN −27/−21 are actually spacing mutations of +1 and +4, respectively, and LSN −32/−21 is an internal deletion of −1, they have been classified as members of the LSN series. These constructs were not part of a systematic spacing analysis, and they were more informative when interpreted as LS mutations of specific genetic elements.

Cell culture and transient transfection assays. HeLa cell

cultures were maintained in Dulbecco modified Eagle medium with 10% calf serum (GIBCO Laboratories) at 37°C in 7.5% CO_2. Transfections were conducted by using modifications of procedures previously described (6, 7, 40; T. McClanahan, unpublished observations). At 16 to 24 h before transfection, cells were plated out at 4 × 10^6 cells per 10-cm dish. These plates are split in a ratio of 1:2 during the transfection procedure. For each construct, a 125 mM $CaCl_2$–1× BBS {BES [N,N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid]-buffered saline}–DNA precipitate was allowed to form just prior to transfection by using the methods of Chen and Okayama (6). 1× BBS is 25 mM BES–140 mM NaCl–0.75 mM Na_2HPO_4, pH 6.95. The volume of the DNA–$CaCl_2$–BBS mixture was determined by the number of plates to be generated. This volume can be calculated by multiplying the amount of DNA to be used per plate (10 μg per plate) by the number of transfected plates that are desired and dividing the result by the DNA concentration of the mixture (20 μg/ml). While the precipitate incubated at room temperature for approximately 15 min, two plates of cells were trypsinized, pooled, and gently centrifuged (setting no. 3, 3 min; IEC clinical centrifuge). Trypsinization of cells was allowed to proceed until cells were just loosened. The cell pellet was resuspended with the DNA–$CaCl_2$–BBS mixture. Following a 15-min incubation with occasional swirling to avoid clumping, the resulting mixture was split to the desired number of 10-cm plates containing Dulbecco modified Eagle medium with 10% calf serum supplemented with 6.25 mM $CaCl_2$ and 0.05× BBS to maintain the precipitate. After 16 h at 3% CO_2, plates were washed twice with Dulbecco modified Eagle medium. Each plate was then supplied with 8 ml of Dulbecco modified Eagle medium containing 10% calf serum and placed at 37°C in 7.5% CO_2. After 24 h, cells either were harvested or were sealed with Parafilm and immersed in a 42°C water bath, or were incubated in the presence of 30 μM $CdSO_4$, or were incubated in the presence of 5 mM L-azetidine-2-carboxylic acid and then harvested. Induction times are indicated in the figure legends.

RNA isolation and analysis. Prior to harvesting each plate was washed twice with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 · $7H_2O$, 1.4 mM KH_2PO_4) containing 0.5 mM EDTA. The cells were lysed in 200 μl of lysis buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris [pH 8.6], 0.5% Nonidet P-40, 0.015% Macaloid), and the nuclear pellet was discarded. To each supernatant, 40 μl of 6× proteinase K buffer (6× proteinase K buffer is 1.2 M LiCl, 100 mM Tris hydrochloride [pH 8], 100 mM EDTA, 1.2% sodium dodecyl sulfate) and 100 μg of proteinase K were added, and the mixture was incubated at 45°C for 2 h. After one phenol-chloroform-isoamyl alcohol (25:24:1) extraction and one chloroform-isoamyl alcohol (24:1) extraction, RNA was precipitated with an equal volume of isopropanol and quantified.

End-labeled S1 probes were prepared by using standard procedures (20). CAT probes, 5′ end labeled at the EcoRI site in the CAT gene, contain sequences extending to an SphI site at either −100 or −188. Protection of correctly initiated HSP-CAT transcripts generates a 400-nucleotide (nt) fragment. Two different NEO probes were used. For most experiments, the NEO probe was 5′ end labeled at the NcoI site NEO gene. This probe extends to −146 and protects 746 nt of correctly initiated transcripts. For the heat shock time course in Fig. 3A, a 400-nt 5′-end-labeled NciI fragment which protects 320 nt of correctly initiated transcripts was used. The probe for the endogenous HSP70 transcripts,

5′ end labeled at the NcoI site (41), protects 575 nt of correctly initiated transcripts. Hybridization conditions, S1 digestions, and electrophoresis on 4% polyacrylamide gels were performed as described previously (40, 41). Bands corresponding to correctly initiated transcripts were quantified by using an LKB laser densitometer. HSP-CAT levels for a given construct were normalized to the corresponding HSP-NEO levels and expressed as a percentage of the levels obtained with a WT construct under the same conditions. The formula used was {[($CAT_{test}/NEO_{test})_n$]/[($CAT_{WT}/NEO_{WT})_n$]} × 100%, where CAT_{test} is the level of HSP-CAT transcripts for the promoter being tested, NEO_{test} is the HSP-NEO level in that same sample, CAT_{WT} is the level of HSP-CAT transcripts for the similarly treated LSN WT sample, NEO_{WT} is the HSP-NEO level in the same LSN WT sample, and n denotes the cell treatment.

RESULTS

Experimental design. We have utilized a transfection approach to identify sequences in the human HSP70 promoter that respond to multiple forms of stress and to examine the interactions between the stress-responsive sequences and the basal promoter elements. To ensure that the level of expression of a given transfected promoter construct is indicative of the promoter being tested and not due to technical inconsistencies, two experimental considerations, the transfection vector and the transfection conditions, have been developed accordingly.

The transfection vector depicted in Fig. 2 contains the CAT test gene and, in opposing orientation, the NEO gene regulated by 74 bp of the human HSP70 promoter. LS mutations, SM, and ID, each in the context of a −188 promoter, and LSP and 5′ deletions (Δ5) were constructed as described in Materials and Methods and by Williams et al. (40) and shuttled into a promoter cassette upstream of the CAT gene. The promoter-containing NdeI-MstII restriction fragments from each of these constructs were shuttled into the NEO-containing vector (such constructs are designated with an N) upstream of the CAT gene, generating the LSN, SMN, IDN, LSPN, and Δ5N series, respectively. Thus, all of the promoter mutations can be analyzed in a common vector background.

The covalently linked NEO gene served as an internal control for the integrity of the transfected DNA, RNA yield, and transfection efficiency. The 74 bp of human HSP70 promoter which regulate the NEO gene lack the sequences required for stress responsiveness (43). Therefore, we expect HSP-NEO expression to be unaffected by stress.

The transient transfection conditions were developed to yield sets of equivalently transfected plates (for specific details, see Materials and Methods). Prior to their final plating, trypsinized HeLa cells are transfected in suspension. The effectiveness of this procedure depends on four parameters: number of cells per plate, duration of trypsinization, amount of DNA per plate, and concentration of DNA in the transfection mixture. Thus, by using these modifications, several plates of cells derived from a single transfection can be obtained.

Kinetics of stress responses. The analysis of promoter mutations consisted of examining the expression of each construct under normal cell growth conditions and following heat shock, exposure to metal ions, and incubation with amino acid analogs. We first examined the stress response of a construct LSN WT containing WT human HSP70 promoter sequences to −188, and the results are presented in
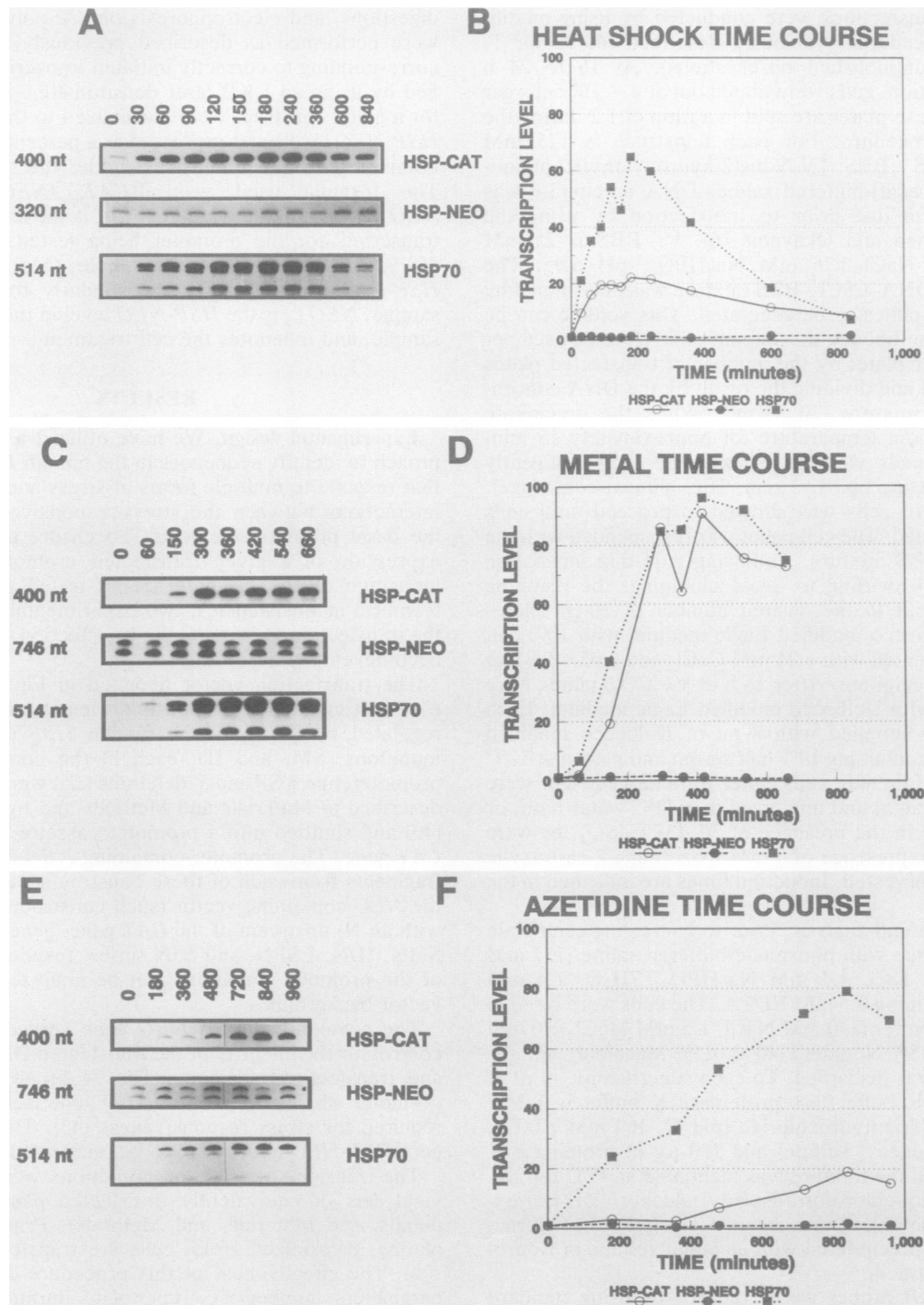
FIG. 3. Response of LSN WT to various forms of stress. (A) HeLa cells transfected with LSN WT as described in Materials and Methods were incubated at 42°C for the indicated times (minutes). S1 protections were performed by using cytoplasmic RNA. The 400-nt protection of *HSP-CAT* transcripts, the 320-nt protection of *HSP-NEO* transcripts with the *Nci*I-labeled NEO probe, and the 514-nt protection (top band) of endogenous *HSP70* transcripts are shown. The gels were quantified by using densitometry, and the transcription levels relative to the corresponding values at 0 min are presented in panel B. (C) Cells transfected with LSN WT were incubated in the presence of 30 μM CdSO₄ for the indicated times (minutes). *HSP-CAT* and *HSP70* protections are as in panel A. The 746-nt protection (lower band) of *HSP-NEO* transcripts was obtained by using an 892-nt *Nco*I-labeled NEO probe (upper band). Quantification of the results in panel C is presented in panel D. (E) Cells transfected with LSN WT were incubated in the presence of 5 mM L-azetidine-2-carboxylic acid for the indicated times (minutes). Protections of the *HSP-CAT*, *HSP-NEO*, and *HSP70* transcripts are as indicated for panel C. The results were quantified and are presented in panel F.
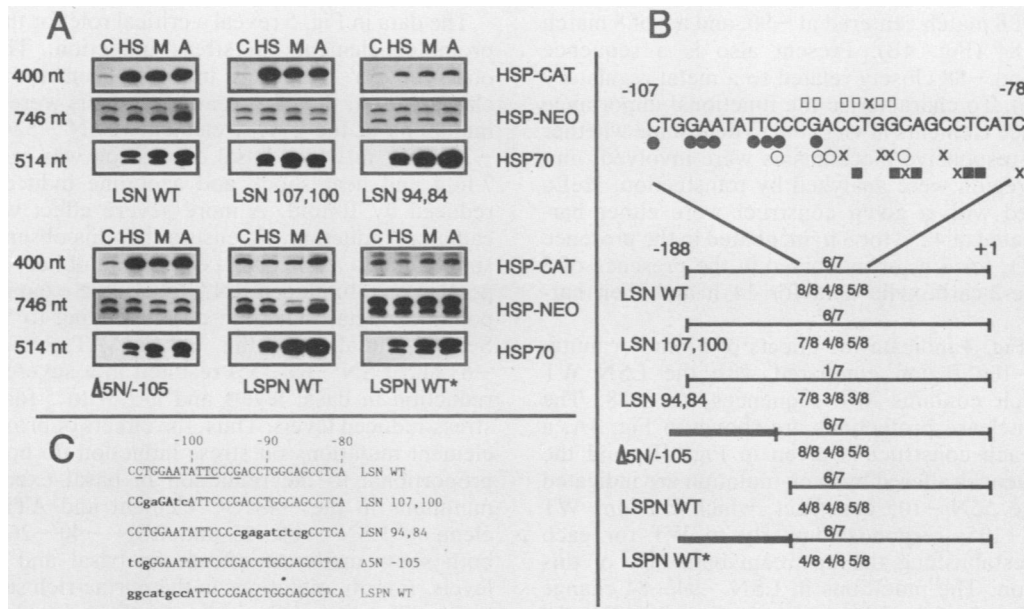
FIG. 4. RNA analysis of HSE mutation constructs. (A) Analysis of cytoplasmic RNA expressed from WT and mutated promoter constructs. HeLa cells transfected with the indicated constructs were either untreated (control [C]), incubated at 42°C for 3 h (heat shock [HS]), incubated in the presence of 30 μM CdSO₄ for 7 h (metal [M]), or incubated in the presence of 5 mM L-azetidine-2-carboxylic acid for 14 h (amino acid analog [A]). S1 analysis was performed as in Fig. 3C, and protections of *HSP-CAT*, *HSP-NEO* (lower band), and *HSP70* (top band) transcripts are shown. (B) Top, *HSP70* promoter sequences from −107 to −78. Matches to the 8 of 8, 4 of 8, and 5 of 8 HSEs are indicated by filled circles, open circles, and filled squares, respectively. Matches to the MRE are denoted by open boxes. Mismatches are indicated by an x. Bottom, Schematics of mutation constructs. Each fit to the MRE is indicated above the construct. The fits to each HSE are indicated below the construct. The thick line denotes pBR322 sequences from position 2297 to position 2066. (C) Nucleotide sequences of mutation constructs. The relevant sequences of the promoter mutations are shown. Mutations are depicted by lowercase letters. The linker regions are indicated by boldface letters. The 8 of 8 HSE is underlined.

Fig. 3. Cytoplasmic RNA was analyzed by S1 protection by using end-labeled probes for *HSP-CAT*, *HSP-NEO*, and endogenous *HSP70* transcripts. Sets of plates for each time course were derived from a single transfection suspension mixture, ensuring that all of the plates for a given time course were equivalently transfected.

The effects of a prolonged heat shock are shown in Fig. 3A and are quantified in Fig. 3B. *HSP-CAT* RNA levels, as measured by the presence of the 400-nt protection, were induced greater than 20-fold between 2 and 4 h of a continuous 42°C heat shock and were maintained at high levels through 14 h. The kinetics of *HSP-CAT* RNA accumulation during heat shock parallel those of the endogenous *HSP70* gene, which are indicated by the levels of the 514-nt protection. The two smaller protections also observed with the endogenous *HSP70* probe probably result from protection of other *HSP70* genes (13). However, only the 514-nt protection was observed with *HSP70* RNA synthesized in vitro from one of the cloned *HSP70* genes (41) (data not shown). *HSP-NEO* levels, as measured by the presence of the 320-nt protection, remain constant throughout the time course. These data confirm the absence of heat shock-responsive sequences in the *HSP-NEO* promoter. Furthermore, in the context of the transfection vector, no enhancerlike activity was observed between the two *HSP70* promoters. In a different context, HSEs have been shown to possess inducible enhancerlike activity (4). The absence of enhancer activity may be due to the lack of multiple HSEs in the *HSP-CAT* promoter or to a distance dependence.

The effects of chemical inducers were also examined. The response to 30 μM cadmium sulfate is shown in Fig. 3C and is quantified in Fig. 3D. The response to the proline analog L-azetidine-2-carboxylic acid (5 mM) is shown in Fig. 3E and

is quantified in Fig. 3F. Maximal *HSP-CAT* RNA levels were attained for metal ions at 7 h and for amino acid analogs at 14 h. This delay, relative to the kinetics of heat shock induction, may be due to their relatively slow uptake or incorporation, as compared with the rapid effects of heat. Alternatively, this could be due to differences in the stress response mechanisms. The kinetics of *HSP-CAT* RNA accumulation paralleled those of the endogenous *HSP70* gene for both chemical treatments. Furthermore, the levels of *HSP-NEO* transcripts, as measured by the 746-nt protection, remained constant throughout the chemical treatments. For all subsequent experiments, a 746-nt protection will be indicative of *HSP-NEO* levels.

The data in Fig. 3 serve several important purposes for the subsequent analysis of promoter mutations. First, establishing the kinetics of inducible RNA accumulation is necessary to ensure that the levels obtained for a given construct represent the maximal stress-induced levels for each construct. Second, the comparisons of the inducible *HSP-CAT* and *HSP70* RNA levels reveal that transfection vector-borne constructs are regulated properly. Third, the constant level of *HSP-NEO* expression during all three stress treatments validates its use as an internal control.

Stress induction requires a single HSE located at −100. Analysis of the upstream region of the human *HSP70* gene reveals sequences that match the 14-bp HSE consensus at −190 and −100. The sequences required for heat shock and metal responsiveness were mapped previously to a 39-bp fragment between −107 and −68 by an analysis of stably transfected 5′ deletion mutations in the *HSP70* promoter (43). The overlapping partial matches to the HSE consensus at −190 were shown to be nonessential (43). Within the 39-bp segment are an 8 of 8 match to the HSE consensus, an

overlapping 4 of 8 match centered at −90, and a 5 of 8 match centered at −84 (Fig. 4B). Present also is a sequence between −95 and −88 closely related to a metal regulatory element (10, 16). To characterize the functional importance of these sequence elements in vivo and to determine whether multiple stress-responsive mechanisms were involved, mutations in this region were analyzed by transfection. HeLa cells transfected with a given construct were either harvested or incubated at 42°C for 3 h, incubated in the presence of 30 μM CdSO₄ for 7 h, or incubated in the presence of 5 mM L-azetidine-2-carboxylic acid for 14 h and then harvested.

The data in Fig. 4 indicate the effects of promoter mutations in the −100 region compared with the LSN WT construct, which contains WT sequences to −188. The results of S1 nuclease protections are shown in Fig. 4A, a schematic of each construct is given in Fig. 4B, and the nucleotide sequences altered by each mutation are indicated in Fig. 4C. The Δ5N/−105 construct, which contains WT sequences to −105, responds similarly to WT for each inducer, thus establishing the upstream boundary of this regulatory region. The mutations in LSN −94/−84 change the 8 of 8 HSE to 7 of 8, reduce both the 5 of 8 and 4 of 8 HSEs to 3 of 8, and also reduce the 6 of 7 match to the metal-regulatory element (MRE) to 1 of 7. These mutations reduce HSP-CAT levels to approximately 40% of WT for all three forms of stress.

Crucial sequences for stress induction were identified by using the LSPN WT construct, which contains WT sequences to −100 and an MRE-like element. This construct changes the 8 of 8 match in the HSE to 4 of 8 and results in the complete loss of all three forms of stress induction. Therefore, the resulting 4 of 8 match together with the remaining 4 of 8 and 5 of 8 matches is insufficient to confer stress responsiveness. Identical results were obtained with the LSPN WT* construct, which is similar to LSPN WT except that the upstream flanking vector sequences have been altered to make it equivalent to a Δ5N/−100 deletion construct. This construct was generated to ensure that the loss of inducible expression observed for LSPN WT was not due to minor differences in the vector. Finally, the LSN −107/−100 construct alters four bases overall but only the substitution at −102 affects a consensus base. These alterations had no effect on inducible expression. Thus, we conclude that heat shock and metal ion and amino acid analog induction of the human HSP70 gene are mediated by a single HSE centered at −100.

Interactions between HSF and the basal transcription complex. Having localized the sequences mediating stress inducibility, we proceeded to investigate the role of the basal promoter elements in the stress response. The basal promoter elements constitute a major difference between vertebrate and invertebrate heat shock promoters. We examined the effects of mutations in the proximal basal elements on three forms of stress responsiveness, and a representative set of results is presented in Fig. 5A. The LSN constructs used in these assays contain LS mutations in the context of a −188 promoter which retains the −100 region HSE required for multiple stress induction. A schematic of each construct is given for each data set in Fig. 5A, and the sequences altered by each mutation are shown in Fig. 5B. For each set of experiments, HSP-CAT RNA levels were normalized to HSP-NEO levels as described in Materials and Methods and the values are presented below the HSP-CAT panels as the percentages of the corresponding WT levels.
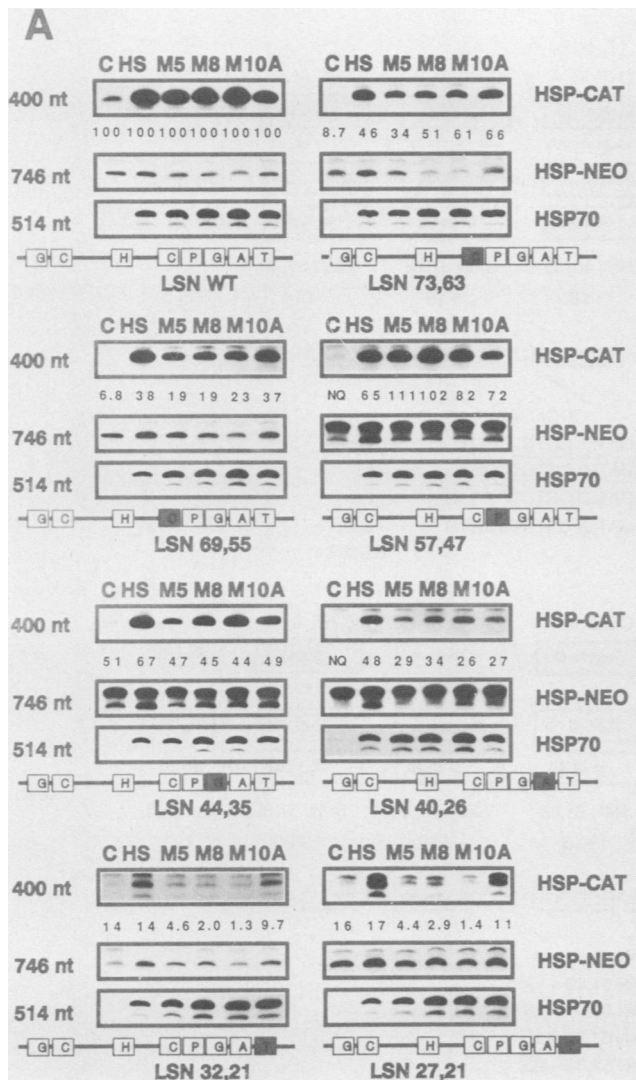
The data in Fig. 5 reveal a critical role for the HSP70 basal promoter elements in stress induction. The phenotypes observed for mutations in these elements fall into four classes. First, the most dramatic effects were observed with mutations in the TATA element (LSN −32/−21 and LSN −27/−21), in which basal expression was reduced by 6- to 7-fold and heat shock and azetidine induced levels were reduced by 10-fold. A more severe effect was noted with cadmium induction. To ensure that this observation was not simply due to an incorrect estimation of harvest time for the peak in metal-induced RNA levels, we examined three time points of metal induction corresponding to 5, 8, and 10 h. Second, mutations in the −67 CCAAT element (LSN −73/−63 and LSN −69/−55) resulted in a severe 11- to 15-fold reduction in basal levels and a 2.5- to 5-fold reduction in stress-induced levels. Thus, the effects of proximal promoter element mutations on stress induction do not appear to be proportional to the reduction in basal expression. Third, mutations in the −48 GC element and ATF- or AP1-like element (LSN −44/−35 and LSN −40/−26, respectively) both show moderate effects on basal and stress-induced levels. Fourth, mutations in the purine-rich sequences (LSN −57/−47) have only minimal effects on both basal and inducible expression.

Examination of metal ion-induced levels reveals an interesting difference between the stress responses. When a proximal promoter element mutation results in a reduction in basal expression, metal-induced levels are reduced relative to the heat- and azetidine-induced levels for the given construct. This effect is most severe with the TATA element mutations in which metal inducibility is nearly abolished. However, when basal expression is unaffected, for example LSN WT and LSN −57/−47 (Fig. 5A and Williams et al. [40]), metal induction is observed at levels equivalent to heat- and azetidine-induced levels. Thus, although the three stress responses require the same sequences in the −100 region for inducibility, our results suggest that differences in the stress response mechanisms exist at the level of basal promoter element requirements.

In summary, the results from the basal element LS mutation experiments suggest that factors bound to the stress-responsive sequences at −100 interact with the basal transcription complex. Stress-induced levels are reduced to different extents depending on which basal element is mutated. The effects are most severe for TATA mutations, followed by ATF/AP1, GC, and CCAAT mutations.

Rotational independence between basal promoter elements. The requirement for both basal promoter sequences in the region proximal to −74 and stress-responsive sequences in the −100 region raises questions as to the nature of the interactions between these domains and with the general transcription machinery. For this reason, we examined the basal and stress-induced transcription of the human HSP70 gene as a function of the spacing between proximal promoter elements. Insertion positions that minimize the disruption of adjacent potential genetic elements were chosen. Parental deletions were chosen such that insertions would approximate integral multiples of half helical turns. Thus, these insertions would either preserve or greatly alter the relative angular orientation of the adjacent promoter elements.

The most proximal set of spacing mutations, consisting of insertions of 4, 12, 16, 15, and 19 bp in the −40 region, is shown in Fig. 6A. By inserting approximately 0.5, 1.0, 1.5, 1.5, and 2.0 helical turns, respectively, between the GC element and the ATF- or AP1-like element, we can examine the requirement for a unique alignment between factors

FIG. 5. Effects of basal-element mutations on stress responsiveness. (A) S1 analysis of cytoplasmic RNA. HeLa cells transfected with the indicated LSN constructs were either untreated (C); incubated at 42°C for 3 h (HS); incubated in the presence of 30 μM $CdSO_4$ for 5, 8, or 10 h (M5, M8, and M10, respectively); or incubated in the presence of 5 mM L-azetidine-2-carboxylic acid for 14 h (A). S1 analysis was performed as described in the legend to Fig. 3C, and protections of *HSP-CAT*, *HSP-NEO* (lower band), and *HSP70* (top band) transcripts are shown. In some cases, readthrough protections are observed with the CAT probe above the central band in the *CAT* panels. Multiple transcription initiation sites corresponding to the lower band in the *CAT* panels are observed for the TATA mutations, LSN −32/−21 and LSN −27/−21. These additional initiation sites have been observed previously (12, 40). The gels were quantified by using laser densitometry. *HSP-CAT* levels were normalized to *HSP-NEO* levels and expressed as a percentage of WT levels under the same conditions by using the formula in Materials and Methods. The results of the quantification are presented as the six values below the *CAT* panels. (NQ, Not quantifiable for technical reasons; see Williams et al. [40] for basal levels of similar constructs.) A promoter schematic similar to that in Fig. 1 is given for each construct. Abbreviations for genetic elements are as described in the legend to Fig. 1. A filled box denotes mutation of the corresponding genetic element. (B) Sequences of mutation constructs. The relevant sequences of the promoter mutations are shown. Mutations are depicted by lowercase letters. The linker regions are indicated by boldface letters. A CCAAT element (−67), a GC element (−48), an ATF- or AP1-like element, and a TATA element (−28) are underlined.

bound to sequences upstream of −40 and factors bound to sequences downstream of −40. The benefit of having two mutations like SMN −40/−42 +16 and SMN −37/−42 +15 with approximately the same size insertion but constructed with different deletion endpoints is that questions of sequence requirements versus spacing requirements can be addressed.

The data in Fig. 6A are from a representative set of experiments in which the −40 region spacing mutations were used. The data indicate that these insertions are minimally disruptive to overall transcription and have almost no effect on stress inducibility. Each spacing construct displays a basal level that is at least 50% of WT basal levels. Any reduction may be due to the slight alteration of the ATF- or AP1-like element. All of the −40 region spacing mutations were stress induced to a level that is greater than 85% of WT stress-induced levels. Furthermore, the basal and stress-induced expression of this series of constructs showed no periodic orientation dependence.

The other set of basal promoter spacing mutations, consisting of insertions of 4, 12, 16, 16, and 20 bp in the −60 region between the CCAAT element and the purine-rich sequences, is shown in Fig. 6B. These constructs contain insertions of approximately 0.5, 1.0, 1.5, 1.5, and 2.0 helical

turns, respectively. The data in Fig. 6B indicate that these spacing mutations, like those in the −40 region, also have little effect on basal expression or on stress inducibility and show no periodic orientation dependence. Basal levels for each construct are at least 50% of WT basal levels. Heat shock- and azetidine-induced levels are greater than 40% of WT levels. Metal-induced levels are, in some cases, only approximately 20% of WT metal-induced levels. This effect is similar to that observed previously for basal-element mutations (Fig. 5). Thus, the basal promoter complex, although defined as a compact multicomponent complex by previous mutational analyses (12, 40), appears to be adaptable to changes in the relative spacing and orientation of its individual elements. Furthermore, the interactions of this complex with factors bound at the HSE show a similar flexibility with respect to the apparent lack of a requirement for a specific rotational alignment.

Rotational independence between the HSE and the basal promoter. The results from the analysis of the SM in the −40 and −60 regions do not exclude the possibility that factors interacting with the −100 HSE form unique contacts with some feature of the basal transcription complex. Placement of these insertions within the basal promoter may have left some interdomain alignments unaltered. Therefore, we placed insertions of 1, 15, and 19 bp between these two domains (Fig. 7). These constructs rotate the basal transcrip-
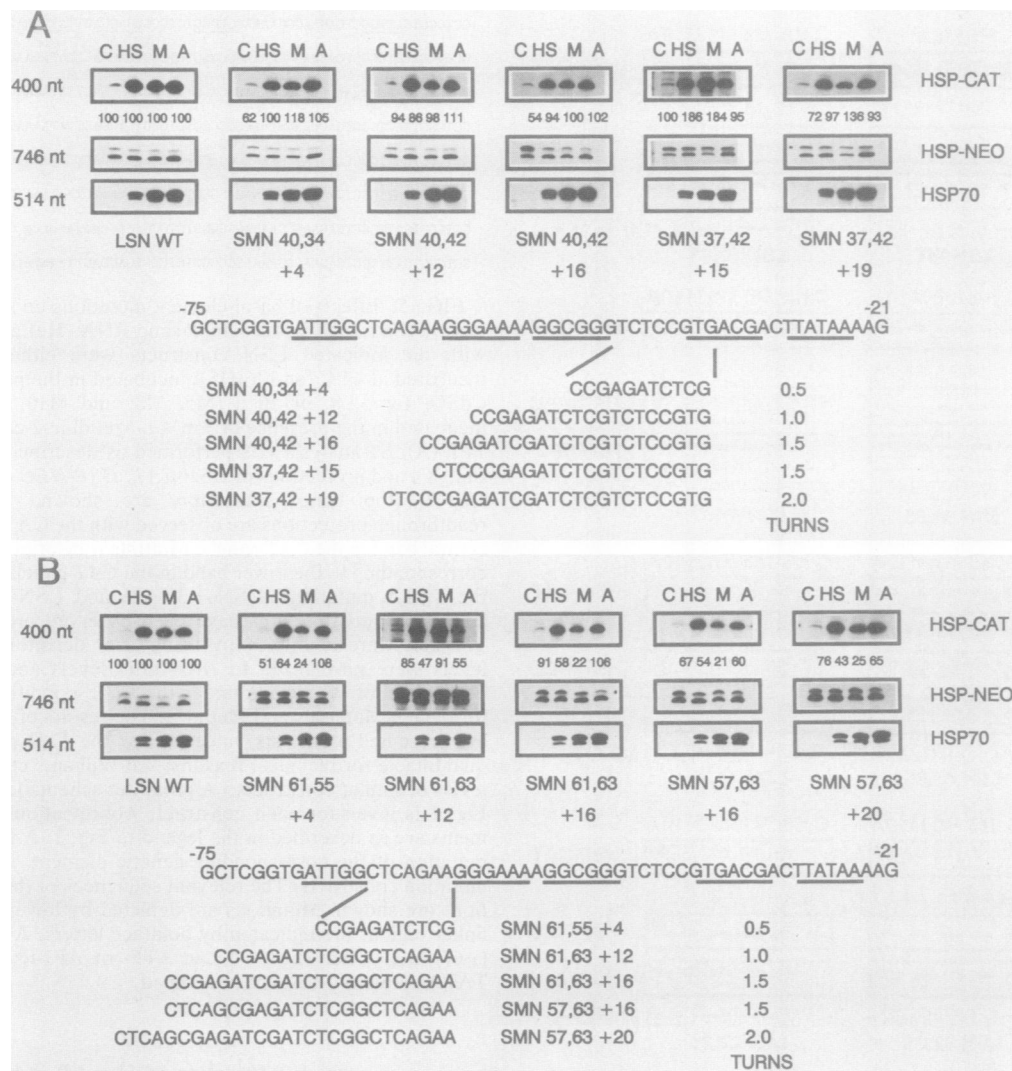
FIG. 6. RNA analysis of stress responsiveness of basal promoter spacing mutation constructs. HeLa cells transfected with the indicated −40 region (A) or −60 region (B) spacing constructs were treated and analyzed as described in the legend to Fig. 4, and the results were quantified. Longer exposures for SMN −37/−42 +15 and SMN −61/−63 +12 were included to compensate for differences in transfection efficiency. The relevant sequences of each spacing mutation are indicated below the data sets. A CCAAT element (−67), a purine-rich element (−55), a GC element (−48), an ATF- or AP1-like element, and a TATA element (−28) are underlined. The approximate numbers of DNA helical turns introduced by each spacing mutation are indicated.

tion complex relative to the HSE by approximately 0, 1.5, and 2.0 helical turns. A representative set of data for this series is shown in Fig. 7. We observed basal levels of greater than 49% of WT basal levels and stress-induced levels that were greater than 100% of WT stress-induced levels. Furthermore, no helical orientation dependence was evident. Thus, the factors that are bound to the −100 region appear to exert their effects on the basal promoter independent of their rotational alignment.

Internal deletions remove requirement for functional basal promoter. We further examined the requirement for basal promoter elements in the stress response by analyzing ID mutations in which the HSE is placed adjacent to proximal promoter elements. IDN −83/−44 and IDN −83/−63 place the 8 of 8 and 4 of 8 matches to the HSE consensus adjacent to the ATF- or AP1-like element and the purine-rich sequences, respectively. IDN −94/−44 and IDN −94/−63

place a 7 of 8 match at the same two proximal promoter positions. These constructs were analyzed as described for Fig. 4, and a representative set of data is presented in Fig. 8.

When the HSE is placed adjacent to the basal promoter elements, the requirement for a fully functional basal promoter is lost. The level of stress induction for IDN −83/−44 and IDN −83/−63 is equivalent to that of WT even though functional basal elements are deleted and basal expression levels are severely reduced. The results for the IDN −94/−44 and IDN −94/−63 constructs are consistent in that induction levels are equivalent to those obtained with a 7 of 8 HSE in its normal position in an otherwise WT promoter construct. These 7 of 8 HSE constructs also confirm the importance of sequences proximal to −94 for HSE activity. In addition, the results from the ID constructs indicate that an HSE does not function as a basal element even when replacing a functional basal element.

FIG. 7. RNA analysis of stress responsiveness of interdomain spacing mutations. HeLa cells transfected with the indicated −80 region spacing constructs were treated and analyzed as described in the legend to Fig. 4. Quantification was as described in the legend to Fig. 5, designating SMN −83/−74 +1, which was equivalent to LSN WT (data not shown), as 100%. The relevant sequences of each spacing mutation are indicated below the data sets. An HSE and a CCAAT element are underlined. The approximate numbers of DNA helical turns introduced by each spacing mutation are indicated.

DISCUSSION

We have assessed the effects of mutations in the human HSP70 promoter by transient transfection to examine the transcription factor interactions that confer basal and stress-induced expression on the human HSP70 gene. We have localized a single genetic element, the HSE at −100, which mediates the response to multiple forms of stress, including heat shock and incubation with heavy metal ions or amino
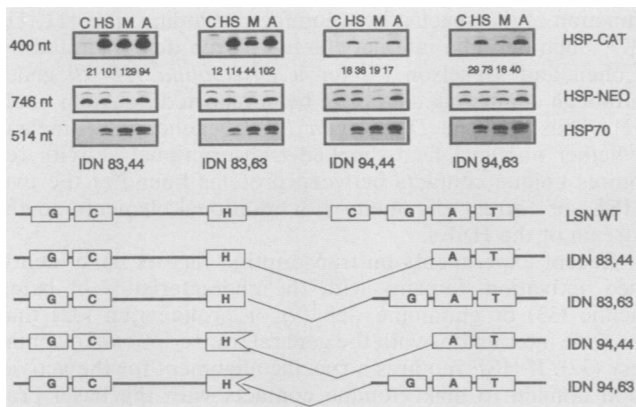

FIG. 8. RNA analysis of stress responsiveness of ID constructs. Top, S1 analysis of cytoplasmic RNA. HeLa cells transfected with the indicated constructs were treated and analyzed as described in the legend to Fig. 4, and the results were quantified. Values are expressed as a percentage of the LSN WT values for the C, HS, M5, and A samples from Fig. 5. These experiments were performed together and were analyzed with the same probes. Bottom, Schematics of mutation constructs. A promoter schematic similar to that in Fig. 1 is given for each construct. Abbreviations for genetic elements are as in Fig. 1. The parental 3′ deletion for IDN −83/−44 and IDN −83/−63, Δ3/−83, contains the 8 of 8 and 4 of 8 matches to the consensus (Fig. 4B). The parental deletion for IDN −94/−44 and IDN −94/−63, Δ3/−94, contains only a 7 of 8 match (formerly 8 of 8). For these constructs, the HSE box has been drawn with a defective edge.

acid analogs. Maximal levels of stress induction require a fully functional basal promoter. Finally, our results reveal that the HSP70 promoter can tolerate substantial spatial and rotational flexibility both within the proximal basal promoter domain and between the proximal domain and the −100 HSE.

Definition of the stress-responsive sequences. We have localized the stress-responsive sequences of the human HSP70 promoter to the distal end of the 39-bp fragment described previously by Wu et al. (43) and have examined the contribution of the 8 of 8, 4 of 8, and 5 of 8 matches to the HSE consensus. However, on the basis of our results with LS and Δ5 mutations, we conclude that the more recent definition of the HSE by Amin et al. (1), Xiao and Lis (46), and Lis et al. (J. T. Lis, H. Xiao, and O. Perisic, in R. Morimoto, A. Tissieres, and G. Georgopoulous, ed., Stress Proteins in Biology and Medicine, in press) as inverted repeating units of the 5-bp sequence NGAAN provides a more accurate description of the HSE for the human HSP70 promoter than the previously used 14-bp (8 of 8) consensus sequence. The revised definition of the HSE allows more accurate predictions as to which potential HSE sequences are likely to be functional. Moreover, it is supported by footprinting studies that support each 5-bp unit as a recognition site for HSF (Lis et al., in press).

On the basis of our data and by using the revised HSE terminology, we conclude that the human HSP70 HSE consists of three contiguous 5-bp units from −105 to −91, as shown in Fig. 9. All three units contain the most highly conserved G, although in the most proximal unit the consensus A has been replaced by a C. The non-stress-responsive LSPN WT (−100) construct has lost completely the distal 5-bp unit. LSN −94/−84, the construct having an impaired stress response, has lost the critical G in its most proximal unit along with the adjacent A but retains the proximal A and weak HSE function. (Compare Fig. 9 and Fig. 4C.)

Our conclusions based on these in vivo studies complement the in vitro results for HSF-HSE interactions summarized in Fig. 9. Mosser et al. (27) have shown that extracts from heat-shocked or metal ion- or amino acid analog-treated cells contain an activity that binds specifically to the HSE. Furthermore, the methylation interference patterns observed by Kingston et al. (18) and Mosser et al. (27) and the DNase I footprint noted by Kingston et al. (18) are centered directly over these three 5-bp units, while a DNase I footprint reported by Morgan et al. (25) for an HSE-binding activity from control HeLa cells appears to extend slightly further downstream.

A common stress-responsive mechanism. A goal of this study was to determine whether heat shock and metal regulation were mediated by the same genetic elements and presumably the same transcription factors or by distinct pathways. Previous deletion studies had mapped these two responses to a 39-bp fragment containing both a potential HSE and a potential MRE. After extending the analysis to include induction by amino acid analogs, we conclude that all three forms of stress are mediated by HSF interactions at the HSE and that the potential MRE is not involved. We base this conclusion on several observations. First, deletion of the distal 5-bp unit in the LSPN WT construct renders the promoter nonresponsive to all three forms of stress even though the potential MRE is left intact. Second, mutation of the 6 of 7 match to the MRE (16) to 1 of 7 in the LSN −94/−84 construct did not result in a selective loss of metal-induced transcription. Third, in vitro studies by Mosser et al. (27) have shown that a factor isolated from

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        ┌─  ●●        ●                    ─┐
  CCCCT  GGAAT  ATTCc  cGAcc  TGGCAGCCTCATC
  -110          ●●                           -78
```
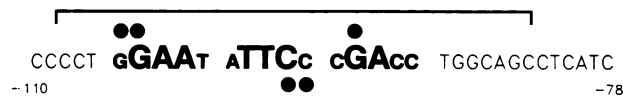
FIG. 9. Summary of human *HSP70* promoter HSE. The human *HSP70* promoter HSE, on the basis of in vivo transfection data, the in vitro transcription factor-binding studies (see text), and the HSE terminology proposed by Lis et al. (in press), consists of the three pentameric units, NGAAN, indicated by bold letters. Matches to the highly conserved GAA (or complement TTC) are indicated with larger letters. The promoter sequences from −78 to −110, which are the sequences protected from DNase I in the footprint observed by Morgan et al. (25), are given. The bracket borders the DNase I footprint observed by Kingston et al. (18). The filled circles summarize the methylation interference patterns (18, 27). Filled circles above the sequences denote interference by methylated guanine residues on the coding strand, and those below the sequences denote interference by methylation at the corresponding guanine residues on the noncoding strand.

metal-induced cells binds to the HSE with the same specificity and contact points as the factor observed in extracts from heat-shocked cells. Fourth, the definition of an MRE has recently been revised by Culotta and Hamer (10) to include a core element and an adjacent GC-like element. The MRE-like sequences in the *HSP70* promoter are related to the GC-like element (16, 43) which by itself is insufficient for metal responsiveness (10). Furthermore, there is virtually no similarity between the metal-responsive sequences of the *HSP70* promoter and the functional core element characterized by Culotta and Hamer (10).

Maximal levels of stress induction require interactions with the basal promoter. The analysis of the effects on stress responsiveness of mutations in the basal promoter region has revealed a requirement for the basal promoter to obtain maximal stress-induced transcription. These results reveal a difference in the organization and regulation between the vertebrate and *Drosophila* heat shock promoters. Since the human *HSP70* promoter has a readily detectable basal level, we have been able to examine the relationship between basal expression and stress induction. In addition to the heat shock element, a fully functional basal promoter is required for maximal response. A simple hypothesis predicts that mutations in basal elements would affect stress-induced transcription to the same degree as they affect basal expression. Surprisingly, the ability of the promoter to tolerate a basal-element mutation under stress conditions is not always proportional to the effect that the given mutation has on basal expression. Mutations in both CCAAT and TATA elements have severe effects on basal expression. However, CCAAT element mutations have only moderate effects on stress-induced transcription, while the effects of TATA element mutations are severe.

The results from the analysis of the internal deletion constructs suggest a possible explanation for the basal- element requirement. When the HSE is in its normal position, there is a strong dependency on an intact basal promoter. However, when the HSE is adjacent to basal promoter elements, WT levels of stress-induced transcription are achieved even in the absence of critical basal elements. We propose that some feature of the *HSP70* promoter basal transcription complex interacts with HSF to optimize its interactions with the general transcriptional machinery. When the HSE is placed adjacent to the basal promoter, this positioning function between HSF and the basal transcription complex is no longer required. Alternatively, the reduction in transcription due to deletion of basal elements is

compensated by relocation of the HSE to a more proximal position. Although an "anchor" function has been proposed for the *Xenopus HSP70* CCAAT element (3), the human *HSP70* CCAAT element could have only a weak anchor function, since mutation of the CCAAT element results in only a moderate reduction in stress-induced transcription. Other systems, such as *Drosophila melanogaster*, appear to overcome this basal-element requirement by either possessing multiple HSEs or positioning an HSE in close proximity to the TATA box or both (5, 36).

We have noticed a difference in the stress response mechanisms when mutations in basal elements are examined. Although a wild-type promoter responds similarly to all three forms of stress, promoter constructs which have a basal-expression phenotype are induced to a lesser extent by metals as compared with induction by heat shock or amino acid analogs. This basal promoter element dependency appears to be the most severe on the TATA element and the least severe on the CCAAT element. These data suggest that the mechanisms which regulate the various forms of stress induction may not be identical. Although the activation of HSF and the HSE-HSF binding interactions may be identical for all three forms of stress induction, metal ion regulation exhibits a stronger dependency for interactions with factors that bind to the basal promoter. It is interesting to note that a different human *HSP70* gene has been reported by Schiller et al. (35) to be nonresponsive to incubation with metal ions. Perhaps the inability of activated human HSF (27) to induce transcription of this gene is because of the lack of essential basal promoter elements.

Rotational alignment-independent promoter interactions. To pursue the anchor function postulated for the basal promoter, we analyzed a collection of spacing mutations specifically designed to test for unique contacts between factors bound to different promoter regions. There are several examples from procaryotes and eucaryotes in which transcriptional activity has been shown to be a periodic function of the spacing between factor-binding sites (11, 21, 37). Such periodic interactions have been demonstrated by Cohen and Meselson (8) for a *Drosophila HSP70* gene, although conflicting data have been reported by Amin et al. (2). Thus, for the *Drosophila HSP70* gene, it is unclear whether maximal heat-shocked transcriptional activity requires unique contacts between proteins bound at the two HSEs or correct alignment with additional elements downstream of the HSEs.

Recent experiments on transcription factors have identified activation domains with the characteristics of being acidic (33) or glutamine rich (9) or proline rich (22) that provide interactions with the general transcriptional machinery (32). If HSF requires a specific alignment for the activation domain to make unique contacts with the basal promoter complex, then we would expect insertion of half turns of DNA to disrupt this alignment and insertion of full turns of DNA to preserve it. The results from our spacing analysis were somewhat surprising. Half-turn and full-turn insertions within the basal promoter in the −40 and −60 regions were only minimally disruptive to basal and stress-induced transcription and revealed that absence of periodicity. Moreover, similar insertions that were placed between the two promoter domains at −80 had no effect. A simple explanation is that multiple HSF proteins are present at the HSE on opposite sides of the helix. Thus, rotation of the DNA at half-turn intervals would still allow a functional interaction. An alternative explanation for the lack of periodicity in the human *HSP70* promoter is that there may be several poten-

tial sites of interaction for HSF at the basal promoter complex and that rotation would simply expose a different redundant target. Thus, although the compactness of the WT human *HSP70* promoter suggests a highly structured multi-component complex, there appears to be a remarkable degree of flexibility with respect to the relative spacing and orientation between the different elements.

ACKNOWLEDGMENTS

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