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Transcription Factor Sp1 Binds to and Activates a Human *hsp70* Gene Promoter

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I investigated the binding of purified transcription factor Sp1 from HeLa cells to the human *hsp70* promoter by DNase I footprinting. Three binding sites were detected within the upstream promoter region, including one located 46 base pairs upstream of the transcription start, between the TATA box and the proximal CCAAT box element. In vitro transcription demonstrated that the proximal site is capable of responding to Sp1-dependent stimulation. These results suggest that Sp1 might contribute to constitutive expression in vivo and might also be involved in the various regulatory responses that affect this gene.

The mechanism by which the expression of eucaryotic genes is regulated by *cis*-acting DNA elements and *trans*-acting protein factors is a subject of considerable importance in cellular and molecular biology. The expression of heat shock genes such as the *hsp70* family, encoding the highly conserved major 70-kilodalton heat shock proteins, is particularly interesting for several reasons. The strong heat shock response of some members of this family represents a convenient system for the study of inducible gene expression, and the *cis*-acting element required for this process is relatively well characterized. In addition to heat shock induction, various other important regulatory responses that affect *hsp70* gene expression have been observed in mammalian cells, including serum stimulation, cell cycle dependence, stimulation by nuclear oncogenes, and elevated expression in transformed cells (1, 22).

Several functional *hsp70* gene copies are present in the human genome, including multiple heat-inducible genes (6, 10). One complete human *hsp70* gene, which appears to be located on chromosome 6 (10), has been cloned and sequenced (32). The expression of this gene under various conditions has been the subject of several studies (24, 33–35). The interaction of sequence-specific DNA-binding proteins from HeLa nuclear extract with *cis*-acting elements of this promoter has also been investigated (21, 26, 36). In this report, I describe work to extend this study of promoter-specific factors that regulate the *hsp70* gene.

Examination of the upstream promoter sequence of this *hsp70* gene revealed short G+C-rich sequences at −46 and −168 that partially resembled the GC-box elements of promoters such as the simian virus 40 early promoter. The GC-box elements of SV40 and many other viral and cellular genes mediate transcriptional activation by Sp1, a sequence-specific DNA-binding protein from HeLa cells (23). Recent work has determined a consensus recognition sequence for Sp1 binding and has demonstrated that there is considerable variation among binding sites from various loci (16). Therefore, I considered it important to investigate the possibility that Sp1 is involved in transcriptional regulation of this human *hsp70* gene promoter. Advances in purification of mammalian DNA-binding proteins by sequence-specific DNA affinity chromatography and other methods have led to highly purified and active preparations of Sp1 (2, 16). I used affinity-purified Sp1 to test directly for interactions with the *hsp70* promoter. I show here that Sp1 binds to multiple sites within the upstream promoter region and demonstrate by in vitro transcription and specific mutagenesis that the most proximal binding site is capable of mediating Sp1-dependent transcriptional stimulation.

Plasmid constructs containing sequences derived from the human *hsp70* promoter (pH2.8, Δ-133, and ΔS-8) were described previously (26). The Δ-133 and ΔS-8 plasmids (with identical upstream sequences) exhibit both strong constitutive and heat-inducible transcription and are therefore considered to be essentially wild type relative to deletion or substitution mutants in the heat shock element or proximal promoter domain. Plasmids Δ5P and LSP44-35 were the generous gift of G. T. Williams and R. I. Morimoto. Δ5P is similar to the previous series of 5′ deletions in plasmid pBR-N/B (26), with the following modifications: *hsp70* sequences upstream of the *Ssp*I site at −102 are deleted, sequences between the *Hin*dIII and *Sph*I sites in the vector are deleted, and the *Eco*RI site in the vector is eliminated. LSP44-35 was created from Δ5P by fusion of matching 5′ and 3′ BAL 31 deletions (9) and contains a *Bgl*II linker replacing the wild-type sequence (Fig. 1B).

HeLa nuclear extracts were prepared and fractionated as described previously (2, 26). Affinity purification of Sp1 and CCAAT box-binding transcription factor (CTF) on synthetic oligodeoxynucleotide columns was performed as described previously (2, 13, 16). Pure Sp1 and CTF (95% homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was prepared by three successive passes through the affinity resin. Sp1 and CTF prepared by a single pass through the resin was approximately 50% homogeneous. In vitro transcription and DNase I footprinting were performed as described previously (26). Transcription was quantitated by densitometry of the autoradiograms.

I examined the binding of Sp1 to the human *hsp70* gene promoter by DNase I footprinting (5). A very active Sp1 preparation, purified by sequence-specific DNA affinity chromatography, was used for this purpose. This preparation was 95% homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The *hsp70* promoter DNA fragment was end labeled at the *Bam*HI restriction site located 150 base pairs downstream of the transcription start site. Three specific protected regions were detected within the upstream promoter region, centered at −46, −167, and −241 (Fig. 1A). Protection of the opposite strand at the same three locations was also observed with a probe end labeled at the *Nco*I restriction site at −270 (data not shown). The extent of the protected regions

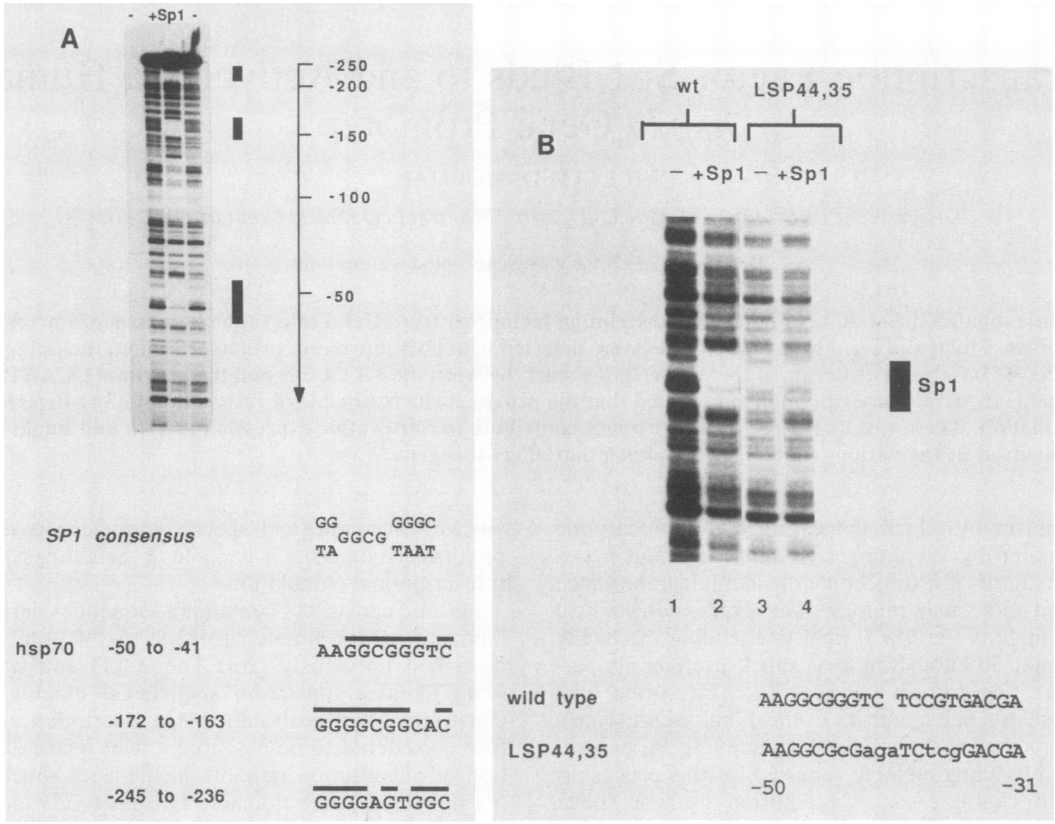


FIG. 1. Binding of Sp1 to the human *hsp70* gene promoter. (A) DNase I footprinting of the human *hsp70* promoter with affinity-purified Sp1. Minus lanes, no protein; center (plus) lane, 5 μl of Sp1. The protein was purified from HeLa nuclear extract as described by Briggs et al. (2), through the fast protein liquid chromatography MonoS column stage, and then chromatographed on a synthetic oligodeoxynucleotide resin containing the decanucleotide recognition sequence for Sp1 (GGGGCGGGGC) (16). This Sp1 preparation contained 1 unit of footprinting activity per μl as defined by Briggs et al. (2). The probe was, per assay, 6 fmol, without carrier DNA, of the pH2.8 *Nco*I-*Bam*HI fragment, 5′ end labeled at the *Bam*HI site (at +150). The sequences of the recognition consensus and three individual *hsp70* promoter binding sites are given below the autoradiogram. (B) DNase I footprinting of a linker-scanner mutation in the proximal GC-box sequence of the human *hsp70* promoter. Lanes 1 and 3, no protein; lanes 2 and 4, 5 μl of Sp1, affinity purified as described for Fig. 2B. The probes were, per assay, 11 fmol, without carrier DNA, of wild-type Δ5P (lanes 1 and 2) or linker-scanner mutant LSP44-35 (lanes 3 and 4). Both probes were *Bam*HI-*Nde*I fragments, 5′ end labeled at the *Bam*HI site (at +150). The sequence of the clustered base substitution in LSP44-35 is indicated below the gel, together with the wild-type sequence.

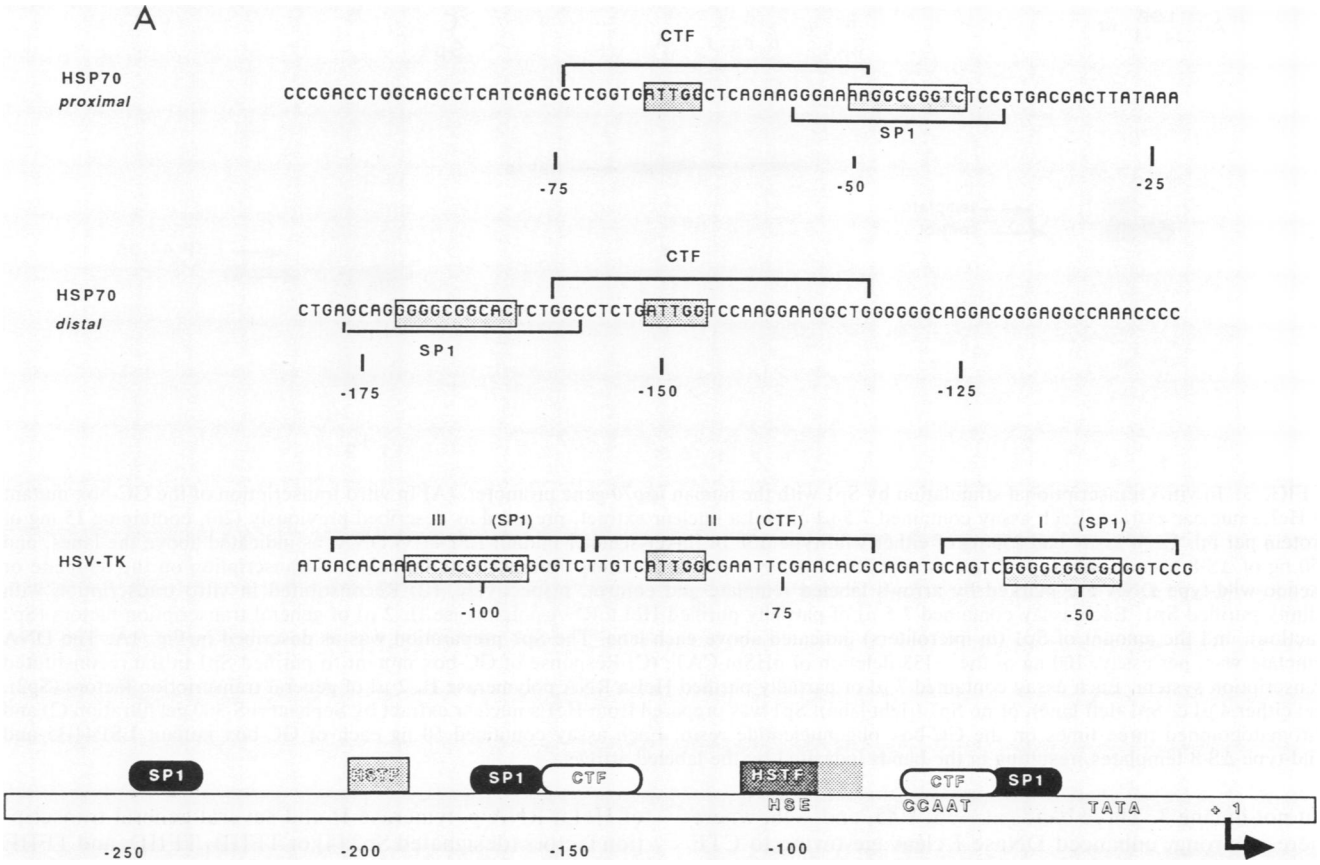
was approximately 16 base pairs, consistent with previous observations of Sp1 binding (16). Each of the binding sites contains a sequence with homology to the decanucleotide consensus recognition sequence (GC box) for Sp1 (16) (Fig. 1A).

Sp1 binding was not clearly seen in previous experiments with less highly purified fractions, although these contained Sp1 (26). This apparently reflects the relatively low affinity of the *hsp70* binding sites, which may require concentrated Sp1 and may also be sensitive to ionic strength and the presence of carrier DNA. It is also difficult to identify the Sp1-binding sites in less pure fractions, because they are close to other, strongly protected sequences such as the CCAAT boxes. As a result, the footprint appears as a continuous protected region, where individual sites are hard to distinguish (see Fig. 4A of reference 26).

The specificity of Sp1 binding to the proximal GC box was also tested by using a mutant with a linker-scanner (clustered base substitution) mutation in this region. This mutant, LSP44-35, has a 3-base-pair change within the GC box, disrupting two positions where the *hsp70* sequence is in agreement with the preferred decanucleotide consensus for Sp1 binding (Fig. 1A). There are other changes downstream of the GC box, and the linker also results in a 1-base-pair insertion. DNase I footprinting with an LSP44-35 DNA fragment labeled at the *Bam*HI site (+150) demonstrated that Sp1 binding is eliminated or strongly reduced (Fig. 1B).

The Sp1-binding sites at −46 and −167 are each located adjacent to CCAAT-box sequences that have been previously shown to bind the transcriptional activator CTF (26). This refers to the CTF/NF-1 activity purified on the basis of functional transcription and replication assays (13, 29). This highly purified preparation is somewhat heterogeneous with respect to polypeptide composition, although the degree of functional heterogeneity is uncertain (3, 30).

The arrangement of elements resembles other promoters, such as herpes simplex virus thymidine kinase (HSV-TK), which contain both Sp1- and CTF-binding sites (7, 15). It is interesting that in *hsp70* the spacing between the distal CCAAT box (at −147) and the second GC box (at −167) (Fig. 2A) is similar to the spacing between the CTF- and Sp1-binding sites designated as distal sites II and III in the HSV-TK promoter (15). As expected from the HSV-TK precedent, both factors can simultaneously bind to these sites in the *hsp70* promoter (data not shown). However, for the more proximal *hsp70* sites, the GC box (at −46) is



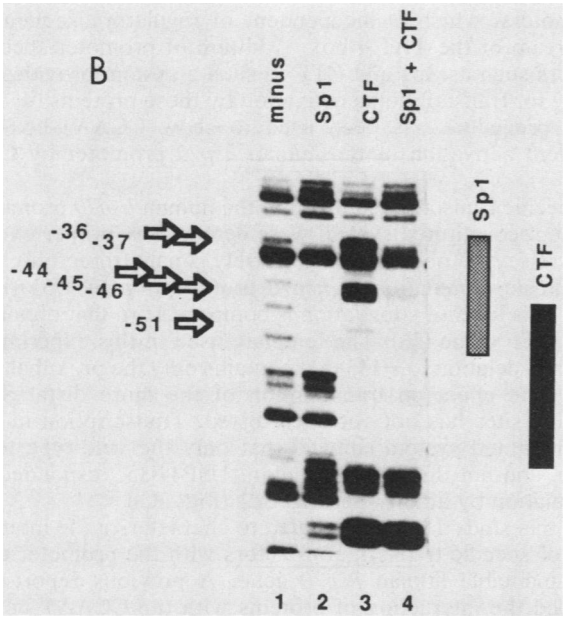


FIG. 2. Topography of CTF and Sp1-binding-site sequences in the human *hsp70* promoter. (A) Comparison with HSV-TK promoter. CTF sites are from reference 26. HSV-TK (distal sites I, II, III) sites are from reference 15. The lower diagram is a summary of transcription factor-binding sites on the human *hsp70* promoter. (B) Mixing experiment with affinity-purified Sp1 and CTF. A heparin-agarose fraction, eluted with 0.3 M KCl, was prepared as described by Briggs et al., (2) and then chromatographed once on two synthetic oligodeoxynucleotide resins containing the GC box and the α-globin promoter CCAAT-box recognition sequences, as described previously (13, 16). Assays contained 2.5 μl of the proteins indicated above each lane. The probe was, per assay, 6 fmol, without carrier DNA, of the ΔS-8 *Hin*dIII-*Bam*HI fragment, 5′ end labeled at the *Hin*dIII site (at −135). In lane 4, the two protein fractions were combined before addition of the DNA probe. The positions of Sp1 and CTF binding to the proximal GC-box and CCAAT-box sequences, centered at −47 and −65, respectively, are indicated by the rectangles on the right. The numbered arrows to the left of the gel indicate specific protected and enhanced cleavage bands, characteristic of either Sp1 or CTF binding, that are discussed in the text. Numbers refer to the nucleotide position of each band in the *hsp70* promoter sequence.

located downstream of the inverted CCAAT box, and the spacing between sites is shorter than in HSV-TK. Because of this and the asymmetric position of the CTF footprint with respect to the CCAAT box, there is substantial overlap between the Sp1 and CTF protected regions (Fig. 2A). Therefore, I tested the ability of CTF and Sp1 to bind simultaneously to the proximal CCAAT box and GC box. When both Sp1 and CTF were added, an extended DNase I footprint that covered both the proximal GC box and CCAAT box was observed (Fig. 2B, lane 4). Comparison of this extended footprint with the individual Sp1 and CTF ones (Fig. 2B, lanes 2 and 3, respectively) suggested that both factors were simultaneously bound to the DNA in lane 4. The presence of bound CTF was indicated by the strong protected region, similar in lanes 3 and 4, which is shown schematically by the rectangle labeled CTF. The presence of Sp1 was shown by the pattern near the downstream end of the footprint (toward the top of the figure). The digestion pattern in this area of lane 4 resembles lane 2 (Sp1), but differs from lanes 1 (minus protein) and 3 (CTF). Specifically, the band at position −44 is protected in lanes 2 and 4,

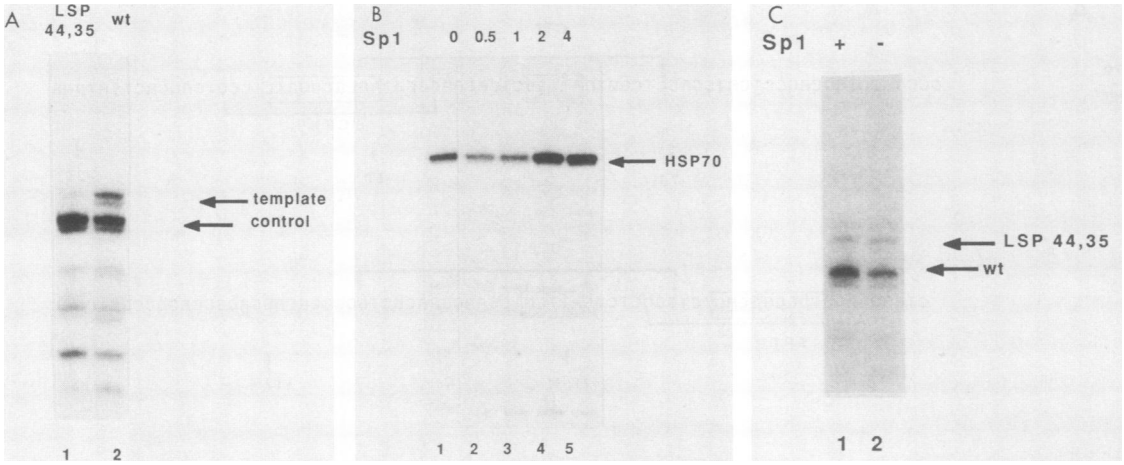


FIG. 3. In vitro transcriptional stimulation by Sp1 with the human *hsp70* gene promoter. (A) In vitro transcription of the GC-box mutant in HeLa nuclear extract. Each assay contained 7.5 μl of HeLa nuclear extract, prepared as described previously (26), containing 15 mg of protein per ml. Each assay had 250 ng of either wild-type Δ5P or linker-scanner mutant LSP44-35 DNA, as indicated above the lanes, and 250 ng of ΔS-8 pseudo-wild-type DNA as an internal control. Bands resulting from accurate initiation of transcription on the template or pseudo-wild-type DNA are marked by arrows labeled template and control, respectively. (B) Reconstituted in vitro transcription with affinity-purified Sp1. Each assay contained 7.5 μl of partially purified HeLa RNA polymerase II, 2 μl of general transcription factors (Sp2 fraction), and the amount of Sp1 (in microliters) indicated above each lane. The Sp1 preparation was as described in Fig. 1A. The DNA template was, per assay, 100 ng of the −133 deletion of pHSm-CAT. (C) Response of GC-box mutant to purified Sp1 in the reconstituted transcription system. Each assay contained 7 μl of partially purified HeLa RNA polymerase II, 2 μl of general transcription factors (Sp2), and either 4 μl of Sp1 (left lane), or no Sp1 (right lane). Sp1 was prepared from HeLa nuclear extract by Sephacryl S-300 gel filtration (2) and chromatographed three times on the GC-box oligonucleotide resin. Each assay contained 50 ng each of GC-box mutant LSP44-35 and wild-type ΔS-8 templates, resulting in the bands indicated by the labeled arrows.

but not in lane 3. Three bands at −36, −45, and −46, where there is strong, enhanced DNase I cleavage owing to CTF binding in lane 3, are eliminated or dramatically reduced in lane 4 when Sp1 is also present. A minor enhanced cleavage band in lane 2 at −37, due to Sp1 binding, is also observed in lane 4. An additional minor enhanced cleavage at position −51 in the CTF footprint disappears when Sp1 is also added. As a consequence of the narrow spacing between the binding sites, the region protected against DNase I cleavage by CTF extends into the edge of the GC box. However, the observation of simultaneous binding of the two factors suggests that CTF does not block access of Sp1 to essential base pairs of its recognition sequence.

The effect of the proximal GC box on in vitro transcriptional activity was tested by using crude HeLa nuclear extract, with the clustered base substitution mutant LSP44-35. This mutant, as well as the wild-type parent plasmid Δ5P, contains the proximal domain of the promoter to −102. The LSP44-35 mutant has at least a fourfold reduction in template activity compared with Δ5P (designated wt in Fig. 3A). The pseudo-wild-type template ΔS-8, which produces a shorter transcript owing to a 9-base-pair internal deletion, was used as an internal control in these assays. Previous observations indicated that a deletion to −54 was slightly more active as a template in crude HeLa nuclear extract than a −47 deletion (26). This difference is also consistent with Sp1-dependent stimulation. The difference in activity between ΔS-8 and Δ5P is probably due to the different lengths of 5′ upstream sequence and perhaps the nature of vector sequences fused to them.

Transcription components from HeLa nuclear extracts have previously been fractionated to develop reconstituted transcription systems for assaying promoter-specific transcription in vitro (4, 31). I investigated the transcriptional effect of affinity-purified Sp1 on the human *hsp70* promoter by using one of these reconstituted systems. A combination of HeLa RNA polymerase II and several general transcription factors (designated Sp2 [4] or TFIIB, TFIID, and TFIIE [31]) provides a low level of accurate initiation at eucaryotic promoters, which is independent of regulatory sequences upstream of the TATA box. Addition of promoter-specific factors such as Sp1 and CTF to such a system provides an assay for transcriptional activation by these proteins (4, 15). This procedure has been used to show CCAAT-box-dependent activation of the human *hsp70* promoter by CTF (26).

Specific transcripts initiated at the human *hsp70* promoter in the reconstituted system were detected by primer extension assay. I observed a relatively small (threefold) but significant stimulation of *hsp70* transcription by Sp1 (Fig. 3B). This level of stimulation is comparable to that obtained with CTF alone (26). The template used in this experiment was a 5′ deletion to −133 that contains only the proximal GC box. The effect on transcription of the more distal Sp1-binding sites has not yet been tested. Transcription in the reconstituted system showed that only the wild-type template, and not the GC-box mutant LSP44-35, responded to stimulation by affinity-purified Sp1 (Fig. 3C).

In this study I have continued to characterize the interaction of specific transcription factors with the promoter of a heat-inducible human *hsp70* gene. A previous report described the interaction of proteins with the CCAAT boxes and heat shock element (26). Here I examined the binding of HeLa cell transcription factor Sp1 to the human *hsp70* promoter by DNase I footprinting. Protection of three specific sites in the upstream promoter region by affinity-purified Sp1 was detected by using fragments that extended from +150 to −270. I investigated the significance of Sp1 binding for transcription by two approaches. First, transcriptional stimulation by affinity-purified Sp1 was demonstrated with a reconstituted in vitro transcription system. Second, a clustered base substitution mutant that alters the proximal

GC box and eliminates Sp1 binding was found to significantly reduce the transcriptional activity of the template in crude HeLa nuclear extracts, as well as in the fractionated system.

The three Sp1-binding sites consist of 16-base-pair protected regions, similar in size to other Sp1-binding sites, and are centered over sequences that are related to the decanucleotide consensus determined for Sp1 recognition (16). The three sites located at −46, −167, and −241 have 70 to 80% homology with the preferred decanucleotide consensus (5′-GGGGCGGGGC-3′) and 80 to 90% homology with the variable form of the consensus (Fig. 1A). By comparison, the Sp1-binding sites in the HSV-TK promoter, distal sites I and III, have 80 to 90% homology with the preferred recognition sequence. Although a 70% match to the 10-base-pair consensus might occur fortuitously in a large fragment, it is unlikely to occur adjacent to the TATA-box sequence. The studies of HSV-TK and other promoters suggest that relatively low-affinity Sp1-binding sites (compared with the simian virus 40 early promoter, for example) can be important for transcription activity, particularly if they are located close to CCAAT-box and TATA-box elements (7, 12, 14, 15). For this reason, it is plausible that the Sp1-binding sites on the human *hsp70* promoter, especially the proximal one, function in a similar fashion.

The in vitro experiments shown above suggest that the proximal *hsp70* GC box is functional for transcriptional activation and show directly that this effect is mediated by the Sp1 protein. Previous work showed that interaction of CTF with the proximal CCAAT box was required for strong constitutive promoter activity (26). The present study indicates that Sp1 may also contribute significantly to the activity of the proximal promoter domain, although it alone is not sufficient to give strong activity if the CCAAT-box element is removed. Significantly, footprinting experiments showed that both proteins can bind simultaneously, despite the very close spacing of their respective consensus sequences and overlap of DNase I-protected regions. Preliminary mixing experiments with reconstituted in vitro transcription indicate that the activity is higher in the presence of both CTF and Sp1 (data not shown), although since not all templates are used for transcription, it is possible that the two factors are not acting simultaneously on the same template. It will be useful in further work to characterize binding cooperativity and functional interactions between CTF and Sp1 with this promoter.

Results of in vivo experiments suggest an additional basal promoter element in the proximal domain, at or near the GC-box sequence. Previous work showed a low level of promoter activity in 293 cells with a −54 deletion mutant that lacked the CCAAT-box element. Transcription was further reduced by deletion to −47, extending into the proximal GC box (34). Linker scanning mutants in the GC-box region have been found to result in a small but detectable depression of promoter activity (8). The magnitude of this effect is consistent with the degree of Sp1-dependent transcription seen here in vitro.

The promoter of this human *hsp70* gene responds to a number of different regulatory stimuli, including heat shock induction (32), serum stimulation (34, 35), cell cycle control (17, 24), and stimulation by nuclear oncogenes such as the adenovirus E1A 13S gene product (18, 28, 33) and polyomavirus large T antigen (19, 20). Biochemical analysis and precise mutagenesis are required to determine whether Sp1 is involved in these processes. The developmental and tissue specificity of expression of this *hsp70* gene copy has not yet been characterized, but Sp1 could affect the pattern of activity. A chicken *hsp70* promoter has a G+C-rich sequence very close to a CCAAT box, although Sp1 binding to this region has not been demonstrated (27). It is interesting that the Sp1-binding site at −167 overlaps a sequence where binding of polyomavirus large T antigen has been detected (20), although the significance of this observation is not clear. Other potential regulatory sites for the enhancer-binding protein AP-2 (25) and cyclic AMP-responsive factor ATF (11) have also been identified and may contribute to the above regulatory phenomena. Extensive purification and characterization of Sp1 (2, 16) have enabled us to examine potential interactions of this factor with the human *hsp70* promoter directly by in vitro binding and transcription studies. This analysis will facilitate further evaluation of the role of Sp1 in concert with other proteins in various aspects of the complex regulation of this gene in vivo.

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