Cooperative Binding of Heat Shock Transcription Factor to the Hsp70 Promoter in Vivo and in Vitro*

(Received for publication, June 29, 1993, and in revised form, October 15, 1993)

Jahanshah Amin†, Mary Fernandez‡, Jayakumar Ananthan†, John T. Lis†, and Richard Voellmy†‡

From the †Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101 and the ‡Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

The minimal promoter of the Drosophila hsp70 gene contains a TATA box and two nonidentical HSE sequences, HSEI and HSEII, that synergistically activate the promoter. We have examined stereospecific alignment and spatial constraints in this promoter. Similar to deletion of HSEII, insertion in the spacer between the HSEs of 1 to 5 or 11 to 14 nucleotides (nt) reduced promoter activity to about 10%. In contrast, HSEI was capable of contributing to promoter activity when the spacer was either shortened by 2 or 4 nt or extended by 6 to 10 or 16 or 18 nt. Hence, half of the possible helical arrangements of HSEs are compatible, whereas the other half are essentially incompatible with efficient promoter function. HSEII was ineffective when its distance to HSEI was increased by more than 18 nt. In vitro, HSEII is a weak and HSEI a strong binding site for heat shock transcription factor HSF, and HSF binds to HSEII cooperatively. To find out whether the above periodicity reflects cooperative binding of HSF in vivo or represents the need of stereosignment for synergistic activation of transcription, the weak HSF binding site HSEII was replaced with the strong binding site HSEI. This substitution greatly attenuated promoter periodicity, suggesting that the periodic effects are caused by cooperative binding of HSF to HSEII, and that stereosignment of HSEs is not required for transcription activation. In agreement, in vitro assays using spacer mutants revealed cooperative binding of purified, recombinant HSF to HSEII with a similar periodicity as observed in vivo. Changing the distance between TATA and the HSEs did not produce promoter periodicity, indicating that stereosignment of these elements is not important.

Heat shock protein (hsp) genes are a class of genes that are present in all cell types examined so far and are typically silent at temperatures at which normal growth occurs but are expressed at exceedingly high levels at elevated temperatures or in cells suffering from other types of stress (1, 2). This regulation appears to include both transcriptional and post-transcriptional components (3). Analysis of hsp gene promoters initially focused on the Drosophila hsp70 gene (4–6), but findings made with this promoter have been extended subsequently to other hsp genes from Drosophila and other organisms (for a review, see Ref. 7). The unusually compact arrangement of sequence elements in the Drosophila hsp70 promoter has permitted the definition of a fully functional, minimal promoter segment (8–11) that comprises 88 nt of 5'-untranscribed and about 30 nt of transcribed sequence. The 5'-untranscribed region contains a consensus TATA box and two non-identical copies of a sequence element referred to as heat shock element (HSE). HSEs were shown to confer heat regulation on the hsp70 promoter (5, 6). Their action on transcription is synergistic; deletion of either HSE reduces transcription by 10-fold or more (12).

Exonuclease III protection (13, 14) and footprinting assays (15) identified a protein, referred to now as heat shock transcription factor (HSF), that binds to HSEs in a heat-induced fashion. Partially purified HSF was found to stimulate transcription in vitro, and, as for transcription in vivo, this effect required the presence of both HSEs in the minimal hsp70 promoter (15, 16). HSF binds in vitro to the upstream HSE (HSEII) with a 12.5-fold higher affinity in the presence than in the absence of the downstream element (HSEI; Ref. 16). Recent experiments with highly purified HSF have revealed that active HSF exists as a homotrimer and that in vitro binding of HSF to extended synthetic HSE sites is highly cooperative (17–19).

HSF binding to the adjacent HSE sites in the minimal hsp70 promoter may participate in different kinds of protein-protein interactions: first, HSF bound to the HSEI site may interact with a second HSF molecule to facilitate its binding to HSEII. Second, HSF molecules bound to the two HSE sites interact, either individually or as a complex, with the transcription machinery, causing the synergistic activation of the promoter (20). Whereas the latter type of interaction may or may not be dependent on the stereosignment of HSE sites with respect to each other and/or to downstream sites, cooperative binding interactions could be expected to require alignment of the HSE sites. Since cooperative binding of HSF to HSEs had already been observed in in vitro assays, we wished to find out whether these interactions could also occur in vivo. To this end, the stereosignment of the two HSE sequences in the minimal promoter was altered systematically by increasing or decreasing the size of the spacer separating them. Analysis of the transient activity of the mutant promoters in Drosophila cells revealed discrete, periodic changes in transcriptional activity, consistent with the occurrence of cooperative binding interactions in vivo. Parallel assays of cooperative binding of HSF in vitro using a similar set of mutant promoters revealed a comparable periodic pattern, supporting this interpretation. To confirm that cooperative binding interactions involving HSF occurred in vivo as well as to find out whether interactions of HSF with the transcription complex may be dependent on stereosignment of one or both HSEs and the TATA box, similar transient expression assays were carried out with two additional series of mutant

* These work was supported by National Institutes of Health Grants GM31125 and GM25232. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Miami School of Medicine, P.O. Box 016129, Miami, FL 33101-6129. Location: R. Bunn Gautier Bldg., 1011 N. W. 15th Street, Miami, FL 33136-1019.

‡ The abbreviations used are: hsp, heat shock protein; nt, nucleotide; HSE, heat shock element; HSF, heat shock transcription factor.
promoters, the first testing the effects on promoter periodicity of the replacement of the weak HSF binding site HSEII with the high affinity HSF binding site HSEI, and the second the effects of changes in the distance between the HSE sequences and the TATA box. The results support the conclusion that cooperative binding of HSF to HSE sequences occurs in vivo and may be an important aspect of the regulation of promoter activity in hsp genes. Furthermore, the data also suggest that stereoealignment of bound HSF molecules and the transcription complex is not required for the synergistic activation of the promoter. We have attempted to integrate these findings in a model of hsp70 transcription regulation that involves interactions between HSF molecules and a post-initiation transcription complex (Ref. 21, and references therein).

MATERIALS AND METHODS

Plasmid Constructions—All constructs were derived from pD88 (12). In this construct, a Drosophila melanogaster hsp70 gene segment that included, following an XhoI linker, 88 nt of 3'-nontranscribed sequence, the entire RNA leader region, and the first seven hsp70 codons (from the hsp70 promoter segment). Hsp70 promoter and P-galactosidase fusions were constructed by insertion of a ScaI site 2 nucleotides upstream of the first HSE, insertion of a ScaI-EcoRI fragment of HSF was blunted and inserted into a filled-in ScaI-EcoRI site of pSP72.

RESULTS

Expression and Purification of Drosophila HSF—HSF-Bac was constructed by insertion of a ScaI site 2 nucleotides upstream of the first HSF codon of Drosophila HSF cDNA (a gift from C. E. Richardson). Recombinant baculovirus was propagated in Spodoptera frugiperda Sf21 insect cells (obtained from A. Wood). Sf21 cells were co-infected with wild type AcNPV baculovirus and HSF-Bac DNA. Recombinant virus was identified visually as blue plaques in medium containing the indicator dye X-gal. Restriction fragment analysis of the HindIII-digested HSF insert of the plaque-lacking the occlusion bodies characteristic of wild type virus. Recombinant virus was then purified by seven rounds of infection and plaque isolation. Identification of recombinant virus was confirmed by Western blot using an anti-HSF antibody raised against a GST-HSF fusion protein or gel-shift assay with lysates infected cells. To purify recombinant HSF, Trichoplusia ni TN5-S1-4 cells (obtained from R. R. Granados) were plated at about 25% confluence in 100-mm dishes (3 x 10^6 cells/plate) and were infected with recombinant virus at a multiplicity of infection of 10. Cells were heat-treated for 20 min at 37 °C 45 h after infection, and nuclear extract was prepared as described previously (1). The hsp70 promoter region was size-selected by a restriction digest with EcoRI, AccI and BamHI and inserted into the SmaI site of pSP72.

Cooperative DNA Binding of HSF

Formation and Purification of the HSF-DNA Complex—The HSF-DNA complex was isolated as described previously (12). HSF-DNA complexes were formed by a 1:1 mixture of purified HSF, end-labeled DNA fragment containing a promoter sequence and unincorporated deoxynucleotides were removed by chromatography on spin columns. DNA binding reactions were performed for 16 h at room temperature. Reactions (30 μl) contained 1 μl of an appropriate dilution of purified recombinant HSF, end-labeled DNA fragment (50,000 cpm corresponding to about 25 fmol of DNA), 1 μg of poly(dI-dC) x poly(dI-dC) and 20 μg of bovine serum albumin in 20 mM Hepes, pH 7.9, 0.1 mM EDTA, 1 mM dithiothreitol, 50 mM disodium EDTA, and 10% glycerol, and 0.1 mM NaCl. Reactions were electrophoresed on 2% native agarose gels in 50 mM Tris, 32 mM boric acid, and 1 mM EDTA.

For determination of relative binding constants the procedure described by Liu-Johnson et al. (26) was employed. Typically, five end-labeled fragments containing spacer sequences of different lengths were mixed together and used in a single DNA binding reaction. Following electrophoresis on a native gel and autoradiography, areas containing complex (complex I contained HSF bound to HSEI, and complex II HSF bound to both HSEI and HSEII as verified by DNase footprinting) and unbound DNA were cut out and placed in Eppendorf tubes. DNA was recovered by freezing the gel slices, and the tubes were vortexed for 20 s and centrifuged for 20 min at 4 °C in an Eppendorf Microfuge. Supernatant solutions were phenol-chloroform-extracted, and DNA precipitated by ethanol. Air-dried DNA was dissolved in formamide sample buffer and electrophoresed in a 5% acrylamide-urea gel (approximately equal amounts of cpm were loaded). A betascope was used to quantitate the intensities of the bands in the autoradiographs. Relative binding constants of different fragments n and m were calculated by using the equation K_n/m = (C/D_n)/(C/D_m), where C is the amount of labeled DNA in complex II and D the amount of labeled DNA in the unbound fraction. Values are averages of at least three independent experiments.

RESULTS

The Minimal Hsp70 Promoter—The basic construct, D88, used in this study contains a 350-nucleotide-long hsp70 gene fragment. The 404-nt segment was standardized to the 270-nt fragments.
The functional 5' boundary of HSEII lies at -87 (in the first imperfect NGAAN motif; Ref. 12). The sequence element GAGAGA that partially overlaps HSEII (-74 to -69) represents a binding site for a general Drosophila transcription factor that has been shown to be required for the normal expression of a number of genes (28, 29). Although this factor binds the GAGAGA motif at the boundary of HSEII in the hsp70 promoter (30), it is not known to play a role in the activity of this promoter.

**Measurements of Promoter Activity**—Activities of mutant genes were compared with that of D88 (or construct OS in the experiment in Fig. 4) in a transfection assay. Briefly, D. melanogaster S3 cells were transfected with construct D88 or its derivatives, and after overnight incubation at room temperature, the cells were placed in a 37 °C water bath for heat treatment or incubated further at room temperature. Actinomycin D was added 15 min after the beginning of heat treatment to heat-treated and not heat-treated cultures to block mRNA synthesis, and the cultures were incubated at 37 °C for an additional 105 min prior to measurement of β-galactosidase activity. A high level of β-galactosidase activity was measured in D88-transfected heat-treated (exposed to heat prior to the addition of actinomycin D) cells, whereas not heat-treated cells had at least 20-fold lower activity (see caption to Fig. 1). As indicated by the results of multiple parallel assays, transfections were highly reproducible within experiments. None of the mutant promoters employed in this study were constitutively active (non-heat shock values omitted). To test whether the β-galactosidase assays accurately reflected the level of transcription, expression from some of the mutant promoters was also analyzed by parallel RNase protection assays (see examples in Fig. 1C). The intensity of the 400-nt protected fragment from transfected genes, standardized relative to the 270-nt protected fragment from endogenous genes, was found to closely parallel the β-galactosidase activity measurements.

**Periodic Variation in Promoter Activity Caused by Systematic Changes in HSEII/HSEI Spacer Length**—To investigate the importance for promoter function of the relative positions of HSEII and HSEI that are 23 nt apart in the wild type hsp70 promoter (center-to-center distance), the spacer region between the HSEs was shortened by deletion or lengthened by insertion, at 1- or 2-nt intervals. Fig. 1B shows the wild type construct D88 and a set of derivatives containing insertions or deletions within the spacer region. Care was taken to avoid the inclusion of any in-phase NGAAN or closely related motifs (GAG, CTC) in the altered spacer regions. Also, the GAGA sequence was

---

**FIG. 1. Variation of hsp70 promoter activity as a function of decreasing or increasing length of the spacer region between HSEII and HSEI.** A, β-galactosidase activities in heat-treated D. melanogaster S3 cells transfected with construct D88 or mutants listed in B. Values are relative to the activity of D88 and represent means from three to six independent experiments ("Materials and Methods"). The standard deviation is indicated for each measurement of mutant promoter activity. Non-heat shock activities of cells transfected with D88 or any of the mutants were 0.05 or less (not shown). B, relevant nucleotide sequences of the hsp70 promoter segments of wild type construct D88 and mutants. HSEII and HSEI sites are boxed, nt conforming to the consensus NGAAN motif are underlined, and spacer sequences separating these elements are shown below. Insertions are denoted by +, and deletions by −, followed by the number of nt inserted or deleted. C, RNase protection assay of S3 cells transfected with D88, +4 or +4a (Table 1). Transfected cells were heat-treated, and cytoplasmic RNA was prepared as described under "Materials and Methods." Results obtained with two different probes are shown (P1, 450 nt; P2, 530 nt). Below the figure are relative amounts of hsp70 RNA. For comparison, β-galactosidase expression levels of the same constructs, measured in parallel cultures, are given in the bottom line.
preserved. Results of activity assays of these mutants are shown in Fig. 1A: promoters with 2- or 4-nt deletions exhibit high relative activity. Insertion of 1 to 5 nt greatly reduces gene expression (note that an activity value of 0.1 reflects the level of activity of a mutant lacking HSEII entirely). Activity is largely restored when the insertion size is increased to 6–10 nt. The configuration produced by an 11-nt insertion is only weakly functional, whereas configurations resulting from 12 to 14-nt insertions are essentially nonfunctional. It appears that promoter function is rescued partially when the insertion size is increased by 16 or 18 nt. These results demonstrate periodic variation in promoter activity, suggesting the existence of protein-protein interactions between HSF molecules binding to the two HSE sites and/or between bound HSF molecules and other factors. Also compatible with this interpretation is the observation that the synergistic transcription effect of HSEII is strongly dependent on its distance from HSEI. Insertion of 10 nt reduced activity by at least 50%, and the synergistic effect was abolished by insertion of 20 nt.

Insertions within the spacer region, while affecting its length, also cause sequence changes that may affect its flexibility. Since the HSEII and HSEI sites are only a short distance apart, these differences in the properties of the spacer regions may influence binding of and subsequent interactions between HSF molecules at the two sites. To find out whether the observed periodic changes in promoter activity occurred largely as a function of the number of nt inserted in or deleted from the spacer region, the effects of differences in the nucleotide sequence and composition of the spacer region on promoter function were tested. Promoters with spacers of different nucleotide sequence were prepared for a number of insertion sizes (see examples, in Table I). It was observed that differences in spacer sequence caused variation in promoter activity at most insertion sizes. The most drastic relative changes were seen with +1 and +5 insertions that apparently can produce configurations of HSEs intermediate in character between fully functional (insertion sizes 0 and +6) and nonfunctional (insertion sizes +2 and +4) arrangements. It may not be surprising that the formation of these intermediate configurations is critically dependent on the nucleotide sequence of the spacer. It was also observed that insertion mutant +4a was more than twice as active as other +4 insertions. This may be due to the unusual nature of the nucleotide sequence in the 4a spacer (a long stretch of alternating G and C residues) that may be capable of assuming a non-B DNA conformation. Since overall the differences in promoter activity were small, they do not affect the validity of our observation of spacer length-dependent periodic changes in promoter activity. In fact, the data obtained with mutants containing alternative spacer sequences, of which examples are shown in Table I, reveal a periodicity almost identical to that observed with the original set of mutants shown in Fig. 1A.

The +1 deletion reducing promoter activity to about 10% of D88 represents an anomaly considering that larger deletions exhibit much higher activities, and that in the −1 deletion the HSEs are similarly aligned as in the much more active +9 insertions (Fig. 1A). In the −1 deletion but not in D88, the NGAN element (that may serve as a functional component of HSE in lieu of NGAAN; note that the corresponding HSEII inversion mutant in Fig. 5 has the element NGTGN) at −71 is included with the NGAAN motif. At 71, the HSEI binding site for HSF may have been effectively extended preventing binding of HSF to HSEII. To test this possibility the −71 NGAN element was changed to NGCCN in mutant −1GCC. Consistent with the hypothesis, this construct was considerably more active than the original −1 deletion (Table I).

**Table I**

| Nt inserted or deleted | Nucleotide sequence of spacer region | Promoter activity relative to D88 (rounded off) |
|------------------------|-------------------------------------|-----------------------------------------------|
| −1                     | AGCCCC                            | 0.1 ± 0.02                                    |
| −1(a)                  | AGGATC                             | 0.1 ± 0.02                                    |
| −1(GCC)                | CCCGCC                             | 0.6 ± 0.05                                    |
| 0                      | AGCCCC                             | 1.0                                            |
| +1                     | AGCCCC                             | 0.2 ± 0.05                                    |
| +1(a)                  | AGATATC                            | 0.1 ± 0.02                                    |
| +2                     | ACCCCCGC                          | 0.1 ± 0.03                                    |
| +2(a)                  | AGCCCGTC                          | 0.1 ± 0.04                                    |
| +3                     | AGCCCCGGC                         | 0.1 ± 0.04                                    |
| +3(a)                  | ACCCCGCGC                         | 0.1 ± 0.00                                    |
| +4                     | ACCCCGAGTC                        | 0.1 ± 0.02                                    |
| +4(a)                  | AGCCCCCGGC                        | 0.2 ± 0.04                                    |
| +4(b)                  | AGCCCCGGCGC                       | 0.1 ± 0.01                                    |
| +5                     | AGGCGCGCGCGC                      | 0.1 ± 0.03                                    |
| +5(a)                  | AGCCCTGAGTC                       | 0.4 ± 0.02                                    |
| +9                     | AGCCCTGCGGCGGCGG                  | 0.5 ± 0.07                                    |
| +9(a)                  | AGCCCTGATTAGATC                   | 0.3 ± 0.01                                    |
| +9(c)                  | AGCGAAATTAGATC                    | 0.5 ± 0.07                                    |
| +10                    | AGCCCTGATGCGGCGG                  | 0.5 ± 0.03                                    |
| +10(a)                 | ACCCGAGCCGCGGCGG                  | 0.5 ± 0.1                                     |
| +11                    | AGCCGAAATTATTCGCCCC              | 0.1 ± 0.03                                    |
| +11(a)                 | AGCTCGAGCCGCGGCGG                | 0.1 ± 0.02                                    |

As active as other +4 insertions. This may be due to the unusual nature of the nucleotide sequence in the 4a spacer (a long stretch of alternating G and C residues) that may be capable of assuming a non-B DNA conformation. Since overall the differences in promoter activity were small, they do not affect the validity of our observation of spacer length-dependent periodic changes in promoter activity. In fact, the data obtained with mutants containing alternative spacer sequences, of which examples are shown in Table I, reveal a periodicity almost identical to that observed with the original set of mutants shown in Fig. 1A.

The −1 deletion reducing promoter activity to about 10% of D88 represents an anomaly considering that larger deletions exhibit much higher activities, and that in the −1 deletion the HSEs are similarly aligned as in the much more active +9 insertions (Fig. 1A). In the −1 deletion but not in D88, the NGAN element (that may serve as a functional component of HSE in lieu of NGAAN; note that the corresponding HSEII inversion mutant in Fig. 5 has the element NGTGN) at −71 is included with the NGAAN motif. At 71, the HSEI binding site for HSF may have been effectively extended preventing binding of HSF to HSEII. To test this possibility the −71 NGAN element was changed to NGCCN in mutant −1GCC. Consistent with the hypothesis, this construct was considerably more active than the original −1 deletion (Table I).

**Alignment of HSE and TATA Sequences Is Not Important for Promoter Function**—The results described above suggest that stereospecific alignment of HSEs may be required to allow either for cooperative binding of HSF to HSEII or for interactions between HSF-bound HSE molecules needed for transcriptional stimulation. Alternatively, they may reflect a need for alignment of HSEII with sequence elements downstream from HSEI. To distinguish between these possibilities, we constructed a series of promoter variants with deletions or insertions of different lengths between HSEI and the TATA box (Fig. 2B). The former interpretation predicts that only monotonous distance effects should be observed except where inhibition occurs due to the closeness of protein binding sites. If, however, the alternative interpretation is true, then periodic variation in promoter activity should be revealed by the experiment. Results showed that the different mutant promoters had similar activities with the exception of those with 4- or 6-nt deletions (Fig. 2A). Hence, there is no need for a specific alignment of HSEs and downstream sequences. The drastic reduction in activity caused by deletion of 4 or 6 nt suggests that these deletions may place HSEI and the TATA box in such proximity as to prevent the simultaneous binding of HSF and components of the transcription complex. Thus, the periodic effects caused by changing the length of the spacer between the HSEs reflects the need for stereoaalignment of the two HSE sites and the HSF molecules binding to these sequences.

**Spacer Length-dependent Variation of Promoter Activity in Vivo and Cooperative Binding of HSF in Vitro Show Similar Periodicity**—Does the observed periodicity in promoter activity reflect cooperative binding of HSF to HSEII? The extent of the role played by cooperative binding interactions between transcription factors is determined by a number of parameters including the affinities of the factors for their respective binding sites. Previous work has shown that in vitro HSF binds HSEII only weakly, but that the affinity of HSF for this element is increased 12.5-fold when HSEI is present (16). To confirm that binding of HSF to HSEII is cooperative and to determine whether this interaction is influenced similarly as promoter activity by changes in the length of the spacer region between the HSEs, we decided to perform in vitro assays of HSF DNA binding. For in vitro DNA binding, purified recombinant Dro-
sophila HSF made in a baculovirus expression system was incubated at limiting concentration with sets of end-labeled fragments that included HSEII, HSEI, and spacer segments of different lengths. HSF-DNA complexes were separated by native gel electrophoresis, and DNA was extracted from complex I containing HSF bound to HSEI and complex II in which HSF bound to both HSEI and HSEII; D, unbound DNA. The autoradiogram shows the different DNA species separated on a sequencing gel. See "Materials and Methods" for details.

**HSE I**

-60

\[\text{CTC\text{GATG}TCCG\text{GAA}}\ldots\text{TATC\text{AAATA}}\]

**Mutant**

-6T

-4T

-2T

D88

+4T

+6T

+8T

+10T

+10aT

+11T

+14T

+16T

+20T

AAAGGGG

AAATGCC

AAATCGG

AAAGCCCG

AAAGAGGG

AAAGCGGGG

AAAGCCTC

AAAGCCTG

AAAGGCCG

AAAGCCG

AAATCGAG

AAAGATCC

AAAGAGG

AAAGATGG

AAAGAGG

\[\text{HSE II for HSEI Provides Evidence for in Vivo Cooperative Binding of HSF} \]

**TABLE II**

| Promoter construct | Relative binding constant (complex II)* | Relative promoter activitya |
|--------------------|-----------------------------------------|-----------------------------|
| -1GCC               | 0.8                                     | 0.6                         |
| D88                 | 1.0                                     | 1.0                         |
| +2                  | 0.3                                     | 0.1                         |
| +3                  | 0.2                                     | 0.1                         |
| +4a                 | 0.4                                     | 0.2                         |
| +6                  | 0.9                                     | 0.7                         |
| +8                  | 1.0                                     | 0.9                         |
| +9                  | 0.6                                     | 0.5                         |
| +10a                | 0.5                                     | 0.3                         |
| +12                 | 0.3                                     | 0.1                         |
| +14                 | 0.4                                     | 0.1                         |
| +16                 | 0.5                                     | 0.2                         |
| +20                 | 0.6                                     | 0.1                         |

* Values from three independent experiments.

aData from Fig. 1 and Table I.

at insertion sizes of +12 and +14, but increased again at larger insertion sizes. These results confirmed the existence of cooperative binding of HSF to HSEII since the relative binding constant changed as a function of the stereoaignment of HSE sequences. There appears to be a close correlation between changes in binding constants and promoter activity for insertion sizes of up to 16 nt. At an insertion size of 20 nt, however, distance appears to have a stronger negative effect on promoter activity than on HSEII binding in vitro, presumably reflecting differences in the conditions of DNA binding in vitro and in vivo. We conclude from these results that cooperative binding of HSF to HSEII adequately explains the periodic variations in promoter activity observed in vivo.

**Swap of HSEII for HSEI Provides Evidence for in Vivo Cooperative Binding of HSF**—If cooperative binding interactions are critical for hsp70 promoter function in vivo, substitution of the weak HSF binding site HSEII with the strong site HSEI should reduce the need for cooperative binding of HSF. In this case, the substitution promoter should be less dependent on the stereoaignment of HSE sites than the HSEII-containing promoter. On the other hand, interactions between bound HSF molecules that may be required for transcriptional enhancement should not be affected by this swap of binding sites. Hence, the extent by which the substitution of the HSEII site reduces the amplitude of the periodic effects should provide a measure of the contribution of cooperative HSF binding to in vivo hsp70 promoter function. We employed transfection assays to test whether substitution of HSEII with HSEI reduced the need for stereoaignment of HSEs. HSEII was replaced with an
the substitution promoter but reduced that of the HSEII-containing promoter whose activity already decreased to 50% following insertion of 3 nt into the spacer region of this mutant promoter only reduced its activity by 55% (not shown) rather than by more than 90% as in the promoter containing the wild type HSEII sequence. This result is analogous to results obtained with the above substitution promoters and supports the conclusion that substitution of HSEII with a strong HSF binding site diminishes the magnitude of promoter periodicity, providing evidence for cooperative binding in vivo of HSF to HSEII. It is interesting to note that replacement of HSEII with HSEI does not increase promoter activity, underscoring the high efficiency of cooperative binding of HSF in vivo.

The Influence of the Orientation of HSEII on Promoter Periodicity—HSEII contains three imperfect and one perfect NGAAAG motif. The latter motif lies in the promoter-proximal half of the HSEII element and may serve as the initial contact site of a cooperatively binding HSF molecule. In the inverted orientation of HSEII, the perfect motif is 5 nt further away from HSEII than in the original orientation. We therefore asked whether inversion of HSEII would change promoter periodicity by 5 nt or place a greater distance constraint on promoter activity than the original orientation. Results of transfection experiments with an appropriate set of inversion mutants (Fig. 5B) indicated that HSEII inversion did not alter promoter periodicity (compare results in Figs. 1A and 5A). The main difference between promoters with inverted and un-inverted HSEII was that the activities of +8 to +10 insertions were considerably lower in the former than in the latter promoters. These results suggest that the initial contact in the cooperative binding reaction may not occur at the perfect motif but perhaps at the proximal end of HSEII. Completion of the binding reaction may become more difficult as the distance between the perfect motif and the contact site is increased.

**DISCUSSION**

Using sets of mutant promoters with altered spacing between HSEs and between HSEs and the TATA box region, we analyzed in detail how minimal hsp70 promoter function depends on the relative helix positions of the two regulatory sequence elements HSEI and HSEII and obtained evidence that cooperative binding of HSF to HSEII occurs in vivo and plays an important role in regulating the activity of this promoter. Depending on the relative helix positions of the HSE elements, cooperative binding of HSF to HSEII can or cannot occur, but once bound to the two elements, HSP molecules appear to be capable of interacting with the transcription machinery independent of the precise location of their binding sites in the promoter.

Three separate lines of experiments provide evidence for cooperative binding of HSF to HSEII in vivo: first, promoter activity changes (with a defined periodicity) as a function of the relative helix positions of the HSE sequences. This suggests the existence of protein-protein interactions between HSF molecules binding to the HSE elements. Second, in vitro DNA binding assays using purified HSF revealed that binding to HSEII is similarly dependent on the helix positions of the HSE sequences as promoter activity in vivo. These results not only confirm earlier studies demonstrating the existence of cooperative binding interactions in vitro but also suggest that the in vivo variation of promoter activity reflects the need for coop-
Promoter periodicity, reflecting the existence of requirements for stereoealignment of factor binding sites and the factors binding to these sites, has been first recognized in studies with an SV40 promoter (34), and similar observations were made with a number of other promoters (32, 34–38). Most pertinent to our experiments is a previous study describing periodicity in the Drosophila hsp70 promoter (39). In these studies concerned mainly with the demonstration of the existence of positional requirements, relative positions of factor binding sites were changed in 5-nt steps. While such extreme changes in relative positions of binding sites are appropriate to uncover evidence for protein–protein interactions, they do not provide any insight into the degree of flexibility inherent in such interactions. There are two components providing flexibility, the DNA underlying and between the binding sites and the binding proteins themselves. We have been able to study this aspect on the example of the adjacent HSE sites in the minimal hsp70 promoter by changing the relative position of the sites by deletion/insertion of 1 nt at the time. We observed a high degree of flexibility as half of all possible relative helical arrangements of HSE sites are conducive to cooperative binding interactions. Furthermore, subtracting the effect of distance per se, interactions appear to occur with similar but not identical efficiencies in all of these relative positions. The remaining arrangements (with the exception of intermediate configurations produced by certain specific spacer sequences in +1 and +6 insertions) are essentially equally nonfunctional, i.e. there is a sharp transition between functional and nonfunctional arrangements of HSE sequences. Hence, at least in the case of cooperative binding interactions involving HSF, these interactions approach the quality of an on/off switch: there essentially appear to be only two types of positional arrangements of binding sites, one type permitting cooperative binding and resulting in nearly complete occupancy of both sites, and the other prohibitive of cooperative binding. It is tempting to speculate that positional requirements such as the ones observed for cooperative binding to HSE sequences could be exploited to provide an additional mechanism of gene regulation: with the proper helical arrangement of two sites required for synergistic activation a promoter may be regulatable by small changes in regional chromosomal superhelicity that may either permit or prohibit cooperative binding of regulatory proteins to the sites. Although there is no evidence that the hsp70 promoter may be regulated by such a mechanism, it is interesting to note that HSEII and HSEI are normally in positions in which rotation of the sites relative to each other by 35° in either direction (partly due to an anomaly discussed under “Results”) greatly reduces promoter activity.

We report herein that hsp70 promoter activity is very sensitive to increases in distance between the HSE sequences. Conflicting results have been reported previously. An earlier study by this group suggested that hsp70 promoter variants with spacers of up to 370 nt in length are active (12). We have subsequently realized that all insertion mutants used in this study included in their spacers the sequence element CGAGAT located 5 nt downstream from HSEII that together with nearby sequences may constitute an additional imperfect HSF binding site with the nucleotide sequence GAGAGAGGATC (underlined are incomplete GAA motifs). To test the hypothesis that the addition of this site artifically strengthened the promoter, we replaced part of the spacer region in one of the mutants such that the sequence in question was changed to CGAGGG. Consistent with the hypothesis, the latter construct was considerably less active than any other mutant in the series (not shown). In a second study reporting similar findings (39), the activity of the promoter may have been influenced by multiple enhancers present in the vector employed that in-

Fig. 5. Hsp70 promoter constructs containing an inverted HSEII sequence. A, β-galactosidase activity assays. B, nucleotide sequences of the HSE-containing promoter region and of spacers used. See Fig. 1 for other details.
Cooperative DNA Binding of HSF

REFERENCES

1. Ashburner, M. and Benner, J. J. (1979) Cell 17, 241–254
2. Sch鲭ner, M. J., Ashburner, M., and Tissières, A. (eds) (1982) Heat Shock: From Bacteria to Man, Cold Spring Harbor Press, Cold Spring Harbor, NY
3. DiDomenico, R. J., Bugasky, G. E., and Lindquist, S. (1982) Cell 31, 593–603
4. Corces, V., Pellicer, A., Axel, R., and Meselson, M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7088–7092
5. Miraulet, M.-E., Southgate, D., and Darzw, E. (1982) EMBO J. 1, 1279–1285
6. Pelham, H. R. B. (1982) Cell 30, 517–524
7. Nover, L., Helmond, D., Neumann, D., Scharf, K.-D., and Schirling, E. (1984) Biol. Zentr. 103, 357–435
8. Amin, J., Meirsl, R., Lawson, R., Klapfer, H., and Voellmy, R. (1985) Mol. Cell. Biol. 5, 197–203
9. Dudley, R., and Tivers, A.A. (1985) Cell 38, 391–398
10. Lee, H., Kraus, L. W., Wolfler, M. F., and Lis, J. T. (1992) Genes & Dev. 6, 284–295
11. Simon, J.-A., Sutter, C. A., Lobel, R. B., Glaser, R. L., and Lis, J. T. (1986) Cell 40, 805–817
12. Amin, J., Meirsl, R., Schiller, D., Dravo, M., and Voellmy, R. (1987) Mol. Cell. Biol. 7, 1055–1062
13. Wu, C. (1984a) Nature 309, 229–234
14. Wu, C. (1984b) Nature 311, 81–84
15. Parker, C. S., and Topol, J. (1984) Cell 37, 273–283
16. Topol, J., Ruden, D. M., and Parker, C. S. (1985) Cell 42, 527–537
17. Perisic, O., Xiao, H., and Lis, J. T. (1989) Cell 59, 797–806
18. Westwood, J. T., Clos, J., and Wu, C. (1991) Nature 353, 825–827
19. Xiao, H., Perisic, O., and Lis, J. T. (1991) Cell 64, 585–593
20. Herschlag, D., and Johnson, B. D. (1993) Genes & Dev. 7, 173–179
21. O’Brien, T., and Lis, J. T. (1991) Mol. Cell. Biol. 11, 5286–5290
22. Amin, J., Ananthan, J., and Voellmy, R. (1988) Mol. Cell. Biol. 8, 3761–3769
23. Sambrook, J., Fritach, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
24. Clos, J., Westwood, J. T., Becker, F. B., Wilson, S., Lambert, K., and Wu, C. (1990) Cell 63, 1085–1097
25. Wu, C., Wilson, S., Walker, D., Davids, I., Paisha, T., Zimmarino, V., and Ueda, H. (1987) Science 238, 1253–1255
26. Liu-Johnson, H.-N., Gartenberg, M. R., and Cruthers, D. M. (1986) Cell 47, 995–1005
27. Xiao, H., and Lis, J. T. (1998) Science 280, 1139–1142
28. Biggin, M. D., and Tjian, R. (1988) Cell 58, 699–711
29. Glaser, R. L., H. G. S., Siegfried, E., Elgin, S. C. R., and Lis, J. T. (1990) J. Mol. Biol. 211, 751–761
30. Gilmour, D. S., Thomas, G. H., and Elgin, S. C. R. (1989) Science 245, 1487–1490
31. Hochschild, A., and Pashme, M. (1986) Cell 44, 681–687
32. Chase, K.-L., Hogge, M. E., and Schwartz, R. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1901–1905
33. Daniloff, S. L., Frederickson, R. V., Taylor, C. Y., and Miyamoto, N. G. (1991) Nucleic Acids Res. 19, 6913–6922
34. Takahashi, K., Vigoreux, M., Budge, M., Wildeman, A., Zenke, M., and Chambon, P. (1986) Nature 321, 121–126
35. Dunn, T. M., Hahn, S., Ogden, S., and Srelet, R. F. (1986) Proc. Natl. Acad. Sci. U. S. A. 81, 5017–5021
36. Gasco, K., Bell, A., Koch, A., Buc, F., and Busby, S. (1990) Cell 62, 733–743
37. Lennard, A. C., Matthes, H. W. D., Ely, J. M., and Chamber, P. (1989) Nucleic Acids Res. 17, 6003–6013
38. Sawadogo, M., and Roeder, R. G. (1986) Cell 43, 165–175
39. Cohen, R. S., and Meselson, M. (1988) Nature 332, 856–858
40. Buratowski, S., Hahn, S., Guarente, L., and Sharp, P. A. (1989) Cell 56, 549–561
41. Chodosh, L. A., Cartwright, R. W., Morgan, J. G., Crabtree, G. R., and Sharp, P. A. (1987) Science 238, 684–688
42. Knoss, J. J., Behbehani, F. J., Masukani, A., and Gronostajski, R. M. (1991) Mol. Cell. Biol. 11, 2946–2951
43. Rulke, D. M., Ma, J., and Pashme, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4232–4236
44. Wu, L., and Berk, A. (1988) Genes & Dev. 2, 403–411
45. Allison, L. A., and Ingles, C. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2794–2798
46. Buettrger, F., Kuehnelt, B., and Diggelmann, H. (1988) Nucleic Acids Res. 17, 3065–3078
47. Pina, B., Brogmeier, U., and Beato, M. (1990) Cell 60, 719–731
48. Ricard-Foy, H., and Harel-Bellan, A. (1987) EMBO J. 6, 2321–2338
49. Scafe, C., Chao, D., Lopes, J., Hirsch, J. F., Henry, S., and Young, R. A. (1990) Nature 347, 491–494
50. Giardina, C., Peric-Ribbo, M., and Lis, J. T. (1992) Genes & Dev. 6, 2190–2200
51. Wu, C. (1990) Nature 346, 864–869
52. Gilmour, D. S., and Lis, J. T. (1985) Mol. Cell. Biol. 5, 2000–2018
53. Gilmour, D. S., and Lis, J. T. (1986) Mol. Cell. Biol. 6, 3884–3898
54. Rosga, A. E., and Lis, J. T. (1990) Mol. Cell. Biol. 10, 6041–6045

Acknowledgments—We are grateful to David Weiss for help with the Figures, to Janet Ingreffea for help with the production of HSF in baculovirus, and to Ruben Baler and Charles Giardina for critically reading this manuscript.