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# Efficient Transcription of a *Caenorhabditis elegans* Heat Shock Gene Pair in Mouse Fibroblasts Is Dependent on Multiple Promoter Elements Which Can Function Bidirectionally

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**A divergently transcribed pair of *Caenorhabditis elegans hsp16* genes was introduced into mouse fibroblasts by stable transfection with vectors containing bovine papillomavirus plasmid maintenance sequences and a selectable gene. The *hsp16* genes were transcriptionally inactive in the mouse cells under normal growth conditions and were strongly induced by heat shock or arsenite. In a cell line with 12 copies of the gene pair, there were estimated to be more than 10,000 *hsp16* transcripts in each cell after 2 h of heat shock treatment. The *hsp16* transcript levels were more than 100 times higher than those of a gene with a herpes simplex virus thymidine kinase gene promoter carried on the same vector. A single heat shock promoter element (HSE) could activate bidirectional transcription of the two *hsp16* genes when placed between the two TATA elements, but the transcriptional efficiency was reduced 10-fold relative to that of the wild-type gene pair. Four overlapping HSEs positioned between the two TATA elements resulted in inducible bidirectional transcription at greater than wild-type levels. The number of HSEs can therefore be a major determinant of the promoter strength of heat-inducible genes in mammalian cells. Partial disruption of an alternating purine-pyrimidine sequence between the two *hsp16* genes had no significant effect on their transcriptional activity.**

All organisms respond to elevated temperature and to various other forms of environmental stress by synthesizing a diverse group of proteins called heat shock polypeptides (HSPs; for reviews, see references 3, 6, 14, 38, 48). Expression of the HSPs is dependent on the rapid and coordinate onset of HSP gene transcription, presumably through a common induction mechanism.

Eucaryotic HSP genes have one or more copies of a 14-base-pair (bp) sequence, referred to as a heat shock promoter element (HSE), upstream from the transcription initiation site (6, 14, 38). The HSE consensus sequence is CNNGAANNTTCNNG (6). The essential role of this sequence in the positive regulation of transcription has been demonstrated by in vivo expression of mutated HSP genes (2, 16, 32, 33, 37) and by the use of synthetic HSEs to make other genes heat inducible (39). The HSEs of the *Drosophila melanogaster hsp70* and *hsp83* genes appear to be specifically protected from nucleases in the nuclei of heat-shocked cells (58, 59). The *hsp70* HSEs are the binding sites for a *Drosophila* heat shock transcription factor (HSTF) that is specifically required for HSP gene transcription in vitro (36, 52).

Transcription of some HSP genes is regulated by hormones or serum factors (21, 54, 57); these inducers operate through promoter elements other than the HSEs (11, 56). Some HSP genes can also be expressed in the absence of environmental stress, particularly during embryogenesis (21, 60).

We have recently cloned and sequenced a cluster of genes from the nematode *Caenorhabditis elegans* encoding two 16-kilodalton (kDa) HSPs that have extensive homology with the small HSPs of *Drosophila* (43, 44). The *C. elegans* genes are in divergently transcribed pairs with very short intergenic regions containing multiple HSEs. We wished to address the question of how the arrangement of the HSEs regulates transcription of the gene pairs and whether sequences upstream from the HSEs have a necessary role in induction of the genes by heat shock or by another stress agent, sodium arsenite.

No practical system is available for comparing the expression of in vitro-mutated genes in *C. elegans*. However, the basic mechanisms of HSP gene induction have been extremely well conserved over long periods of evolution. As a result, HSP genes from a variety of organisms have been faithfully expressed in very distantly related hosts, e.g., *Drosophila hsp70* and the small HSP genes in mammalian cells (4, 8, 13, 33, 37) and *Dictyostelium* heat shock-inducible genes in *Saccharomyces cerevisiae* (9). Therefore, we have been able to use a mouse fibroblast cell line (C127) to express the *C. elegans hsp16* genes. The use of this heterologous host also provides the opportunity to ask whether the *C. elegans* transcription and message processing signals can be recognized and properly utilized by the genetic apparatus of the mouse.

Foreign genes can be introduced into C127 cells on vectors which contain the early transcription unit of bovine papillomavirus (BPV) (15, 29, 46). *Cis*-acting plasmid maintenance sequences (PMSs) within the BPV genome interact with BPV-encoded replication factors to permit extrachromosomal replication of vector episomes (25, 26). Stable copy numbers of BPV vectors can range from 5 to 500 (15, 45, 46; R. Kay, unpublished results). Episomal genes will not be affected by integration site-specific suppression or deregulation of transcription and therefore should have the consistent expression levels required for comparative analyses of gene function.

BPV vectors depend on the direct linkage of the gene under study to an active BPV transcription unit. Thus, the behavior of the gene could be influenced by BPV enhancers (24, 47, 51), transcript readthrough from BPV promoters (15), or altered chromatin states originating in BPV sequences (42). Promoters dependent on a limited supply of

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host transcription factors could also have reduced efficiency or different specificity when present in hundreds of copies per cell. To avoid these potential problems, we constructed vectors containing only the PMSs of BPV, along with a selectable gene. Similar PMS vectors have been shown to replicate episomally in the presence of BPV replication factors supplied in *trans*, but with a much lower average copy number than complete BPV vectors (25).

*C. elegans hsp16* gene pairs on PMS vectors were introduced, along with separate BPV DNA, into C127 cells, where they were stably maintained with copy numbers between 4 and 60. Both *hsp16* genes were transcribed with high efficiency in transfected cells following induction by heat or arsenite. The role of HSEs in modulating promoter strength was demonstrated with a series of rearranged and mutated *hsp16* gene pairs.

## MATERIALS AND METHODS

**Vector construction.** Methods for restriction enzyme digestion of DNA, fragment purification by agarose gel electrophoresis and electroelution, ligation, and cloning in *Escherichia coli* were essentially as described previously (27). Restriction enzymes and polymerases were obtained from Bethesda Research Laboratories, New England Biolabs, or Boehringer Mannheim.

pPN1 was made by excising the 7,300 bp of BPV DNA in pCGBPV9 ΔB5 (29) between the *Cla*I sites at positions 6834 and 7476 in the BPV genomic sequence (10). pPN2 was made by inserting the 365-bp PMS-2-containing *Hae*III fragment from positions 1440 to 1805 in the BPV genomic sequence into the *Eco*RV site of pPN1. pPN3 was derived from pPN2 by excising the *Hin*dIII fragment containing PMS-1. Circularized BPV DNA was made by purifying the 7,945-bp *Hin*dIII fragment of pCGBPV9 (29) which contains the complete BPV genome and autoligating it at low concentration.

**Assembly of the *hsp16* gene pair in pPN1.** The *Eco*RI-*Bcl*I fragment of the right arm of the *C. elegans hsp16-1-hsp16-48* locus inverted repeat (43), which contains the 3′ end of *hsp16-48*, was inserted between the *Eco*RI and *Bam*HI sites of M13mp8. The *Sal*I site in the polylinker was filled in with the Klenow fragment of DNA polymerase I, and a *Bam*HI linker was inserted. The resulting *Eco*RI-*Bam*HI fragment was then excised and ligated with *Bam*HI-cut pPN1 and the *Bam*HI-*Eco*RI fragment which contains the complete *hsp16-1* gene and the 5′ end of *hsp16-48*. The resulting vector has 1,913 bp of *C. elegans* genomic sequence, including a complete *hsp16-1-hsp16-48* gene pair, inserted at the *Bam*HI site of pPN1. The *Bam*HI fragment containing the *hsp16* gene pair was subsequently inserted into the *Bam*HI sites of pPN2 and pPN3.

**Mutagenesis of the *hsp16* gene pair.** pPN1DX was made by digesting pPN1WT with *Xba*I and religating it at low concentration. pPN1IX was made by inserting the purified intergenic *Xba*I fragment into the *Xba*I site of pPN1DX. pPN1MX was made by digesting 1 μg of pPN1DX with *Xba*I, adding 5 volumes of 70 mM sodium acetate (pH 4.5)–300 mM NaCl–2.5 mM $ZnSO_4$–100 U of S1 nuclease (Boehringer Mannheim) and digesting at 37°C for 15 min. The DNA was extracted with phenol and precipitated with 2 volumes of ethanol, and the ragged ends were filled in with the Klenow fragment of DNA polymerase I and ligated. pPN2NF was made by removing the *Hpa*I-*Nsi*I fragment of pPN2WT that contains the 5′ coding region and promoter of *hsp16-1* and replacing it with the equivalent *Hpa*I-*Nsi*I fragment of pPN1IX. To make pPN2RM, pPN2WT was cut with *Rsa*I, exonucleolytically degraded with Klenow fragment in the presence of dATP, dCTP, and dGTP, and digested with 5 U of S1 nuclease as above. This resulted in a modified *Rsa*I site lacking one nucleotide (*Rsa*I*). After extracting and precipitating the DNA as above and digesting with *Nsi*I, the *Nsi*I-*Rsa*I* fragment containing *hsp16-1* was purified and ligated with the *Rsa*I-*Sal*I fragment of pPN1WT containing *hsp16-48* and the *Sal*I-*Nsi*I fragment of pPN2WT containing the neomycin phosphotransferase type II (NPT II) gene. These constructs were verified by sequencing the promoter regions of the *hsp16* gene pairs.

**Transfection.** PN vector (500 ng) and 250 ng of BPV DNA in 250 μl of 250 mM $CaCl_2$ were extracted with chloroform and chilled on ice. An equal volume of cold 2× HBS (1× HBS is 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid], pH 7.1, 1.4 mM $Na_2HPO_4$, 140 mM NaCl, 5 mM KCl, and 6 mM glucose) was added with vigorous mixing, and the resulting precipitate was transferred to a 25-$cm^2$ cell culture flask containing $10^5$ C127 cells in 3 ml of Dulbecco modified Eagle medium with 10% fetal bovine serum (DME-FBS). After 12 h at 37°C, the medium was removed and the cells were glycerol shocked by rinsing in HBS–10% glycerol for 2.5 min and subsequently grown in DME-FBS at 37°C. The antibiotic G418 (Gibco Laboratories) was added to 0.6 mg/ml 48 h after the glycerol shock. Resistant colonies were picked 12 to 15 days after transfection and grown under G418 selection.

**Induction of cells and isolation of nucleic acids.** Adult *C. elegans* nematodes were heat shocked, and their RNA was purified as described previously (44). The heat shock response was induced in subconfluent C127 cell cultures by heating at 42.5°C for 60 or 120 min, followed by 30 min of recovery at 37°C, or by adding sodium arsenite to 100 μM for 90 min, followed by recovery in fresh DME-FBS without arsenite for 60 min.

Cells were rinsed with 25 mM sodium EDTA, pH 7.5–75 mM NaCl and then lysed in the same solution containing 0.5% sodium dodecyl sulfate (SDS) and 100 μg of proteinase K (Boehringer Mannheim) per ml for 40 min at 37°C. The lysate was extracted once with phenol and twice with phenol-chloroform (1:1), and total nucleic acid was precipitated by the addition of 0.1 volume of 3.5 M sodium acetate, pH 5.0, and 2 volumes of ethanol. Pellets were redissolved in 5 mM Tris chloride (pH 7.4)–0.5 mM EDTA. RNA was checked for degradation by examining ethidium bromide-stained rRNA bands after electrophoresis through 1% agarose gels containing 90 mM Tris-borate (pH 8.0)–1 mM EDTA (TBE) and 0.1% SDS and roughly quantified by comparing the intensity of the rRNA bands to those in a known quantity of total RNA.

**Analysis of transfected DNA.** Total cellular nucleic acid was digested with RNase A and restriction enzymes. The DNA fragments were separated by electrophoresis through TBE agarose gels and transferred to Zetaprobe (Biorad) nylon filters in 0.4 M NaOH as described previously (40). Filters were hybridized with nick-translated probes (41) in 50% formamide–5× SSPE (1× SSPE is 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, and 1 mM EDTA)–0.5% SDS–200 μg of heparin (Sigma Chemical Co.) per ml at 42°C for 12 h, washed sequentially for 20 min at 23°C in 5× SSPE–0.1% SDS, 2× SSPE–0.1% SDS, 0.5× SSPE–0.1% SDS, and 0.1× SSPE–1% SDS, then washed for 20 min at 50°C in 0.1× SSPE–1% SDS, and finally washed for an additional 30 min at 50°C in 5× SSPE–0.3% SDS. Filters were exposed to Kodak XAR film with Cronex intensifying

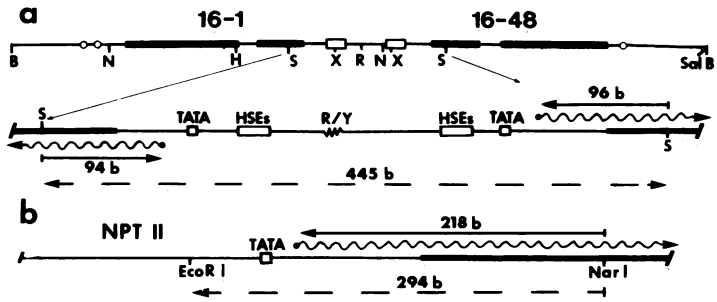


FIG. 1. Structure of the *hsp16* gene pair and the probes used for transcript initiation site mapping. (a) 1.9-kbp *Bam*HI fragment containing the *hsp16* gene pair. Open boxes, Promoters; heavy lines, coding regions; open circles, 3' AATAAA sequences. Abbreviations: B, *Bam*HI site; N, *Nsi*I site; X, *Xba*I site; R, *Rsa*I site; Sal, *Sal*I site; H, *Hpa*I site; S, *Sau*3AI sites flanking the fragment used for transcript initiation site mapping probes; b, bases. (b) Details of *hsp16* gene and NPT II gene promoter regions and structure of the probes used for transcript initiation site mapping. R/Y, Alternating purine-pyrimidine tract. Wavy lines, Transcripts; broken lines, probes; solid lines, length of probe expected to be protected by a corresponding transcript. Transcription initiation sites are those given in references 30 and 43.

screens (DuPont). The amount of vector DNA within a sample of cellular DNA was roughly estimated by comparison to a known amount of purified vector DNA that was transferred to the same filter.

**Transcript mapping and quantification.** A 0.5-pmol amount of 17-mer sequencing primer (Pharmacia) was annealed to 200 to 500 ng of M13mp8 single-stranded template carrying the appropriate insert in 20 mM Tris chloride (pH 7.4)–20 mM $MgCl_2$–20 mM NaCl–2 mM dithiothreitol at 58°C for 60 min. The primer was extended with the Klenow fragment of DNA polymerase I in the presence of 50 μM each dCTP, dGTP, and dTTP and 0.3 μM [α-$^{32}$P]dATP (Amersham Corp.) for 1 min at 23°C. Unlabeled dATP was then added to 50 μM, and the reaction was continued for an additional 5 min. A single cut in the double-stranded DNA was made by adding a restriction enzyme and the appropriate amount of NaCl, and the liberated probe fragment was purified in single-stranded form by electrophoresis through a TBE–urea–40% polyacrylamide gel (27). After excision, the probe fragment was electroeluted in 0.5× TBE–0.05% SDS and precipitated by the addition of 0.1 volume of 3.5 M sodium acetate containing 0.25% polyacrylamide carrier and 2 volumes of ethanol.

For S1 nuclease protection, 100,000 cpm of single-stranded probe was mixed with 1 μg of total nucleic acid in a final volume of 30 μl of 50% formamide–10 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)], pH 6.9–400 mM NaCl–1 mM EDTA, heated to 70°C for 15 min, and then hybridized at 42°C for 12 h. A 170-μl amount of 70 mM sodium acetate (pH 4.5)–600 mM NaCl–2.5 mM $ZnSO_4$ containing 150 U of S1 nuclease (Pharmacia) was then added, and after digestion for 1 h at 37°C the undegraded probe was precipitated by the addition of 30 μl of 100 mM sodium EDTA (pH 8.0)–4 M ammonium acetate–100 μg of tRNA per ml, followed by 230 μl of isopropanol. Samples were separated by electrophoresis in TBE–urea–6% polyacrylamide gels and visualized by autoradiography. Transcripts were roughly quantified by comparing the intensity of probe bands protected by the sample nucleic acid to that of those protected by a known amount of M13mp8 template containing inserts complementary to segments of the probe.

## RESULTS

**Structure of the *hsp16* gene pair.** The *C. elegans* genes that code for HSPs 16-1 and 16-48 are found within the two arms of a 3.8-kbp perfect inverted repeat (43). For expression studies, we subcloned 1.9 kbp of DNA from the right arm of the repeat. This fragment contains single copies of the *hsp16-1* and *hsp16-48* genes in a divergently transcribed pair, separated by 348 bp of intergenic DNA (Fig. 1). A pair of overlapping HSEs and an adjacent TATA element are located upstream from each coding region (Fig. 2). The *C. elegans* HSEs match the general HSE consensus sequence (CNNGAANNTTCNNG [6]) in at least seven of the eight specified bases, except for the distal *hsp16-1* HSE, which has two mismatches. The latter HSE was not identified previously (43) because the more stringent *Drosophila* consensus sequence was used (CTNGAANNTTCNAG [37]). The coding regions are interrupted by single introns of 52 and 55 bp in *hsp16-1* and *hsp16-48*, respectively. The 1.9-kbp fragment includes polyadenylation signal sequences (AATAAA [5, 7]) and an additional 250 bp of DNA downstream from each gene.

**Mutation and rearrangement of the *hsp16* gene pair.** We made several rearrangements in the intergenic region of this wild-type (WT) *hsp16* gene pair (Fig. 2). (i) IX: Inversion of *Xba*I fragment. The 157-bp intergenic *Xba*I fragment of the WT *hsp16* gene pair was inverted. The resulting *hsp16-1* gene has only one HSE adjacent to its TATA element, while the *hsp16-48* gene has three overlapping HSEs. (ii) DX: Deletion of *Xba*I fragment. The intergenic *Xba*I fragment was deleted, leaving a single HSE positioned between the two TATA elements. (iii) MX: Mutation of *Xba*I site. The single HSE in DX was destroyed by a 13-bp deletion, leaving the two TATA elements intact. (iv) NF: *Nsi* site fusion. The two HSEs of the *hsp16-48* gene in WT were directly fused to the single HSE of *hsp16-1* in IX by ligating the upstream *Nsi*I sites. This creates a cluster of four overlapping HSEs positioned between the TATA elements of the two genes; one HSE, with two mismatches to the consensus sequence, is created around the new *Nsi*I site. The NF gene pair is identical to the DX gene pair except for the insertion of three additional HSEs. (v) RM: *Rsa*I site mutation. A run of alternating purines and pyrimidines in the intergenic region of WT was shortened from 10 to 7 bp by deletion of a single base pair within an *Rsa*I site.

**Vector construction.** Matthias et al. (29) have constructed a BPV transfection vector, pCGBPV9 ΔB5, that contains the complete BPV genome, a bacterial origin of replication and phage λ cos site, and the transposon Tn*5* NPT II gene driven by both a bacterial promoter and the herpes simplex virus (HSV) thymidine kinase (*tk*) promoter to allow selection for


FIG. 2. Sequences of the HSEs, TATA elements, and alternating purine-pyrimidine tracts of the wild-type and mutated *hsp16* gene pairs. HSEs are underlined, with dots at mismatches to the consensus sequence. The HSEs are designated by their position in the WT gene pair, e.g., 48P is the *hsp16-48* HSE proximal to the TATA element, 48D is the distal HSE, etc. The bases of the *Xba*I sites in WT, IX, and DX and the bases of the *Nsi*I site of NF are overlined. The alternating purine-pyrimidine tracts have wavy overlines. The base that is deleted in RM is indicated by the open triangle.

kanamycin resistance in *E. coli* and G418 resistance in transfected mouse cells (12). We modified this vector by removing the BPV DNA between the *Cla*I sites (Fig. 3). The resulting vector, pPN1, is 4,400 bp in size and has retained 640 bp of BPV DNA, including PMS-1 (Fig. 3). pPN2 is a modification that also includes PMS-2 as a 365-bp *Hae*III fragment inserted at the *Eco*RV site of pPN1 immediately upstream from the NPT II promoters (see Fig. 5). The relative orientations of the two PMSs are the same in pPN2 and BPV.

The *C. elegans hsp16* gene pairs were inserted into the *Bam*HI site of the PN vectors, with *hsp16-48* adjacent to and transcribed in the same direction as the NPT II gene. The WT, NF, and RM gene pairs were inserted into pPN2, and the DX, MX, and IX gene pairs were inserted into pPN1. The WT gene pair was also inserted into pPN3, a derivative of pPN2 which lacks the 640-bp *Hin*dIII fragment containing PMS-1.

**Transfection.** C127 cells were transfected with 500 ng of supercoiled PN vector and 125 ng of full-length circularized

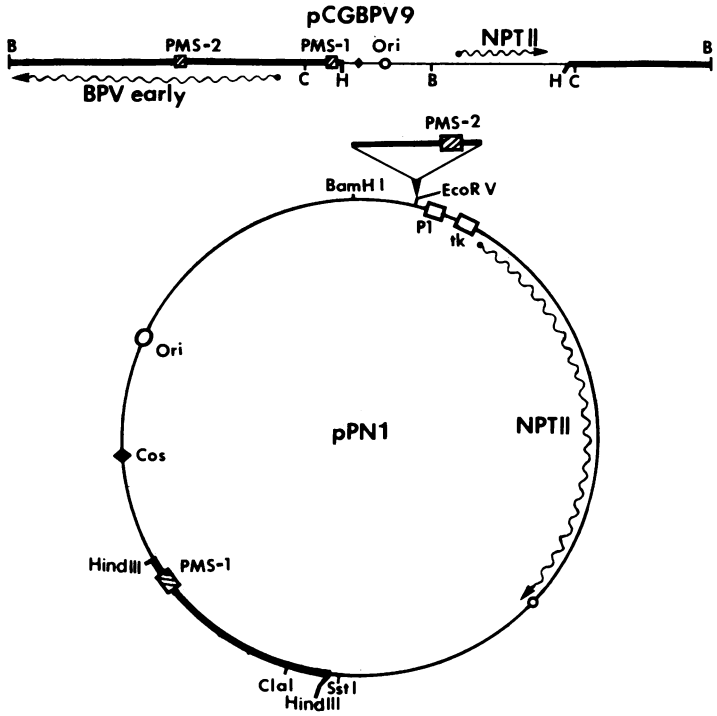


FIG. 3. Structure of transfection vectors. The pCGBPV9 ΔB5 vector is shown linearized at the BPV *Bam*HI site. Heavy lines, BPV sequences; hatched boxes, PMSs; B, *Bam*HI site; C, *Cla*I site; H, *Hin*dIII site; P1, P1 promoter of pBR322; tk, HSV tk promoter; Ori, ColE1-derived origin of replication; Cos, phage λ *cos* site; wavy arrows, transcripts; open circles, polyadenylation signal sequences. The PMS-2-containing insert of pPN2 is shown above the *Eco*RV site.

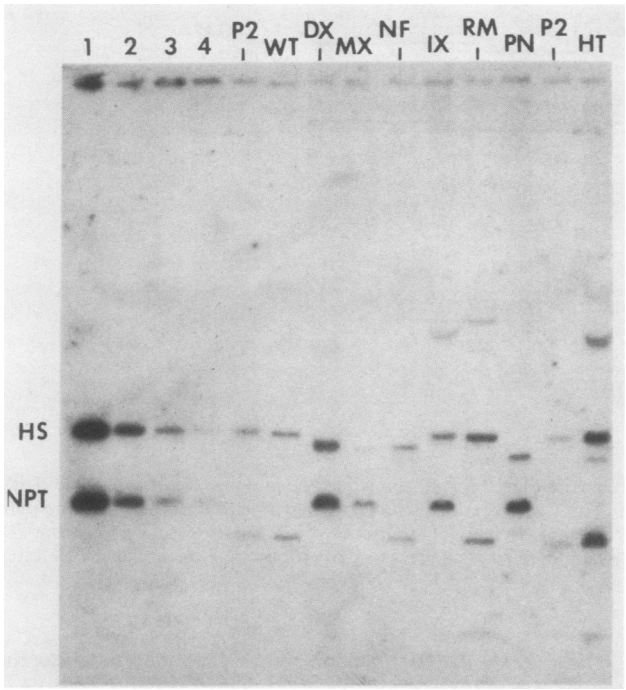


FIG. 4. Structures and copy numbers of the *hsp16* gene pairs and the NPT II genes in the transfected cell lines used for transcript analysis. Approximaely 1 μg of cellular DNA was digested with *Bam*HI and *Nco*I, separated by electrophoresis, transferred to a nylon filter, and hybridized with the nick-translated *hsp16* gene pair *Bam*HI fragment and the nick-translated NPT II *Eco*RV-*Sst*II fragment. Cell lines are designated by the name of the *hsp16* gene pair construct which they carry. HT is the cell line transfected with pPN3WT and used for determining optimal heat shock conditions (Fig. 5c). PN is a cell line transfected with pPN1. Lane P2, 10 pg of *Bam*HI-*Nco*I-digested pPN2WT DNA; lanes 1 through 4, 256, 64, 16, and 4 pg of *Bam*HI-*Nco*I-digested pPN1WT, respectively. One picogram of vector DNA in 1 μg of cellular DNA is equivalent to 0.8 vector copy per cell. HS and NPT indicate the positions of the *hsp16* *Bam*HI fragment and the NPT II *Bam*HI-*Nco*I fragment, respectively.

BPV DNA by the calcium phosphate coprecipitation method (53) and put under selection for G418 resistance. Colonies that were both resistant to the drug and morphologically transformed, and which therefore harbored functional PN vector and BPV genes, were picked and grown as individual cell lines. We typically obtained 10 to 30 of these colonies per transfection and about four times as many resistant but nontransformed colonies.

**Transcriptional activity and structure of transfected genes.** Cell lines from each transfection were heat shocked and screened for transcriptional activity of the *hsp16-1* and NPT II genes. Transcripts were identified and quantified by S1 nuclease protection of continuously labeled single-stranded probes complementary to the 5′ ends of the genes. The probes used and the regions expected to be protected by the transcripts are shown in Fig. 1.

The *hsp16* and NPT II genes were transcribed in all of the transfected cell lines examined, with the exception of the *hsp16* gene pairs that had been inactivated by in vitro mutagenesis. In some cell lines, one or more of the transfected genes were expressed at an abnormally low level, but 40 to 100% of the cell lines that carried a particular *hsp16* gene pair construct expressed the transfected genes with high efficiency and had a similar ratio of *hsp16* to NPT II transcripts (data not shown).

One cell line with optimal and consistent levels of transcription of all transfected genes was selected for detailed analysis of each *hsp16* gene pair construct. The copy numbers and structures of the *hsp16* gene pairs and NPT II genes in these cell lines were revealed by dissecting the genes out of the vectors with *Bam*HI and *Nco*I (Fig. 4). The cell lines transfected with the WT, NF, and RM gene pairs contained smaller NPT II fragments because the pPN2 vector used for these transfections has a *Bam*HI site that is created at the *Hae*III-*Eco*RV junction of the PMS-2 insert proximal to the NPT II promoters (Fig. 3). Differences in the length of the *hsp16* fragment among the cell lines were due to the rearrangements that had been made in vitro. Vector copy numbers were estimated to vary from 4 (MX) to 40 (DX). The copy numbers were reduced 10-fold on average when the BPV DNA was omitted from the transfection; however, contrary to expectations, a BPV-derived PMS did not have to be present within the vector to obtain multiple copies of vector DNA in transfected cells (data not shown).

Most of the PN vector copies were converted to full-length linear fragments by digestion with a restriction enzyme that cut the vector DNA at a single site, but no monomer-sized vector episomes could be detected when the cellular DNA was selectively digested with a restriction enzyme that did not cut within the PN vector (data not shown). The transfected vector DNA was probably in the form of long tandem repeats that were either integrated into the host cell chromosomes or were present as large episomes. Such structures have been observed in C127 cells transfected with either BPV DNA (1) or pCGBPV9 ΔB5 carrying an inserted tryptophan oxidase-chloramphenicol acetyltransferase fusion gene (28). The tryptophan oxidase promoter proved to be equally active in either an episomal or tandemly integrated state.

**Transcription of the WT *hsp16* gene pair.** Transcript levels of the two *hsp16* genes in uninduced, heat-shocked, and arsenite-treated cell lines and in heat-shocked *C. elegans* are shown in Fig. 5. In heat-shocked *C. elegans*, both *hsp16* genes produced a single transcript that was initiated about 20 bp downstream from the TATA element. In mouse cells, the WT *hsp16* genes were transcriptionally inactive under normal growth conditions, except for a minor transcript initiating about 175 bp 5′ to the *hsp16-48* TATA element. The diffuse bands that were also protected by nucleic acid from cells transfected with pPN1 alone were artifacts of the S1 nuclease digestion rather than *hsp16* gene transcripts. No transcripts initiating 3′ to the TATA elements of the *hsp16* genes could be detected in unstressed cells even after a 10-fold-longer period of autoradiography than is shown in Fig. 5. When the cells transfected with the WT gene pair were heat shocked at 42.5°C for 1 h, transcription of both *hsp16* genes was strongly induced. Initiation of transcription occurred at the same position on the *hsp16* genes in both transfected C127 cells and *C. elegans*. Transcription of the transfected *hsp16* genes was induced with the same fidelity and efficiency by arsenite. A minor transcript that initiated 225 bp upstream from the *hsp16-48* TATA element was also induced by heat shock and to a lesser extent by arsenite (Fig. 5b).

By comparison to the amount of probe protected by a known amount of complementary single-stranded DNA, we estimated that about 5 pg of the 16-48 probe was protected by 1 μg of nucleic acid from heat-shocked C127 cells carrying the WT gene pair (Fig. 5b). After compensating for

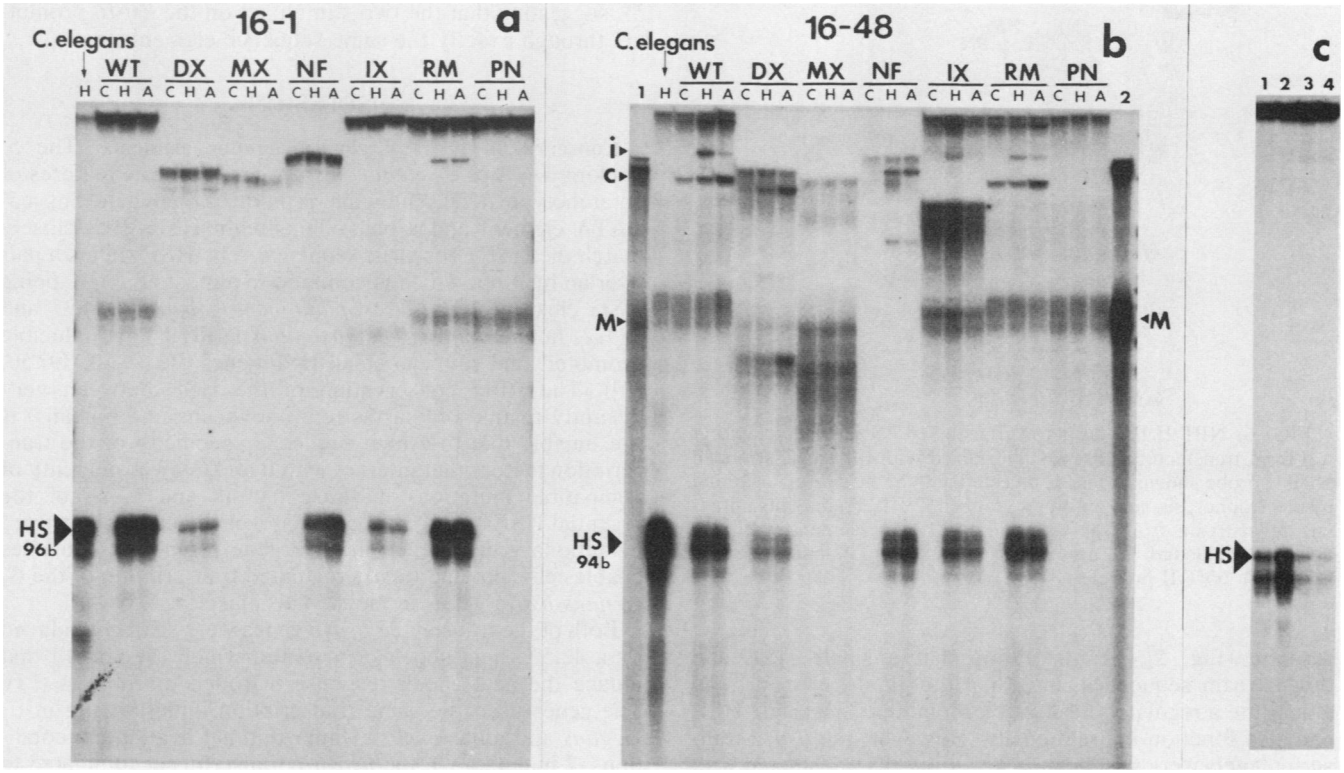


FIG. 5. *hsp16-1* and *hsp16-48* transcript initiation site mapping and quantification. Nucleic acid (1 μg; 0.3 μg from heat-shocked NF) from uninduced (lanes C), heat-shocked (lanes H; 1 h at 42.5°C), or arsenite-treated (lanes A) cell lines or heat-shocked *C. elegans* was hybridized to the *hsp16-1* or *hsp16-48* single-stranded probe appropriate to the transfected construct, digested with S1 nuclease, separated by electrophoresis, and autoradiographed. Cell lines are designated by the name of the *hsp16* gene pair with which they are transfected. PN is a cell line transfected with pPN1. HS, The major inducible transcripts initiating downstream from the *hsp16* TATA elements, with the approximate length of the protected probe indicated; M, bands due to protection of the WT 16-48 probe by 1.1 pg (lane 1) and 22 pg (lane 2) of complementary single-stranded DNA from templates carrying NF DNA inserts; c, constitutively expressed transcript initiating 175 bp upstream from the *hsp16-48* TATA element; i, inducible transcript initiating 225 bp upstream from the *hsp16-48* TATA element. (a) *hsp16-1* probes; (b) *hsp16-48* probes; (c) *hsp16-1* probes: Cells transfected with pPN3WT were heat shocked for 60 min at 42.5°C (lane 1), for 120 min at 42.5°C (lane 2), for 60 min at 43.5°C (lane 3), or for 60 min at 44°C (lane 4).

the full length of the *hsp16-48* transcript (950 bases) and assuming that 2.5% of total cellular nucleic acid is mRNA, the abundance of the *hsp16-48* transcripts was estimated to be about 0.2% of the mRNA in these cells. The *hsp16-1* transcripts were estimated to comprise about 0.3% of the mRNA in the same cells. This is equivalent to 3,000 transcripts per cell. These transcript levels could be raised about fourfold by extending the heat shock period to 2 h (Fig. 5c), but the *hsp16-1* transcript levels were considerably reduced by raising the heat shock temperature 1°C. The *hsp16-1* transcripts comprised about 0.3% of the mRNA in heat-shocked *C. elegans* nematodes. The *hsp16-48* transcripts were about one-half as abundant as the *hsp16-1* transcripts in C127 cells transfected with the WT gene pair, but more than twice as abundant as the *hsp16-1* transcripts in *C. elegans* (Table 1).

There were an estimated 80 NPT II transcripts in each of the cells transfected with the WT gene pair when they were grown at 37°C (Fig. 6 and data not shown). Non-heat-shocked cells were used to measure NPT II transcript levels to avoid any effects of transcriptionally active upstream *hsp16* genes.

The PMS-1-containing fragment in pPN1 and pPN2 includes the recently described upstream BPV enhancer, which is active in the presence of the BPV E2 gene product (51). This enhancer does not have to be present in the vector to obtain efficient expression, because the *hsp16* gene pairs on pPN3, which contains PMS-2 but lacks the PMS-1 fragment, had optimal transcriptional efficiencies similar to or greater than those on pPN2 (Fig. 5c and data not shown).

**Transcription of mutated *hsp16* gene pairs.** In the DX gene pair both *hsp16* genes were still inducible by heat shock or

TABLE 1. *hsp16* and NPT II transcript ratios[a]

| Cells[b] | Transcript ratio | | |
|---|---|---|---|
| | 16-1/16-48 | 16-1/NPT | 16-48/NPT |
| Ce | 0.4 | —[c] | — |
| WT | 1.6 | 40 | 25 |
| DX | 0.8 | 2.5 | 3 |
| MX | — | 0 | 0 |
| NF | 1.7 | 90 | 50 |
| IX | 0.4 | 4 | 10 |
| RM | 1.9 | 55 | 30 |

[a] Proportions of *hsp16* and NPT II transcripts in heat-shocked *C. elegans* and transfected C127 cells were roughly estimated by inspection of the protected probe bands in Fig. 5 and 6, with reference to the amount of probe protected by a known amount of complementary single-stranded DNA. The uncertainty in these estimates is probably in the range of 25 to 50%.

[b] Cell lines are designated by the name of the *hsp16* gene pair construct with which they were transfected. Ce indicates RNA from *C. elegans*.

[c] —, Not applicable.

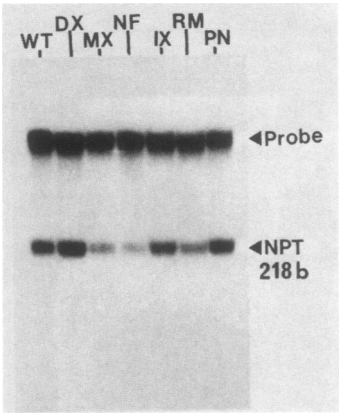


FIG. 6. NPT II transcripts in transfected cells. Nucleic acid (1 μg) from transfected cells was hybridized with the single-stranded NPT II probe shown in Fig. 1, digested with S1 nuclease, separated by electrophoresis, and autoradiographed. NPT, Transcripts initiating downstream from the tk promoter TATA element, with the approximate length of protected probe shown in bases; Probe, full-length NPT II probe.

arsenite (Fig. 5), demonstrating that a single HSE and downstream sequences, including the TATA element, can constitute a regulated heat shock promoter. The single HSE can also function bidirectionally. However, the *hsp16* transcript levels were significantly lower in cells transfected with the DX gene pair than in those transfected with the WT gene pair, both absolutely (Fig. 5) and in proportion to the NPT II transcripts (Table 1). The ratio of *hsp16-1* to *hsp16-48* transcripts was twofold lower for the DX gene pair than for the WT gene pair (Table 1).

The deletion in the MX *hsp16* gene pair that destroyed the single remaining HSE inactivated the two *hsp16* genes (Fig. 5). The NPT II gene was still active in the cells carrying this gene pair (Fig. 6). Therefore, the HSE is required for induction of the genes, and the TATA elements in isolation cannot promote transcription.

The NF *hsp16* gene pair with four overlapping HSEs between the two TATA elements was more efficiently transcribed than the WT *hsp16* gene pair when the transcript levels were normalized to the levels of NPT II transcripts (Table 1). (Note that only 0.3 μg of nucleic acid was used in the NF heat shock samples in Fig. 5, versus 1 μg for the other samples.) The ratios of the *hsp16-1* and *hsp16-48* transcripts produced by the WT and NF gene pairs were equivalent (Table 1). A cluster of multiple HSEs, therefore, can functionally substitute for all the sequences between the intergenic *Xba*I sites in the WT *hsp16* gene pair.

Inverting the intergenic *Xba*I fragment (IX) resulted in a 10-fold reduction in *hsp16-1* transcription and a 2-fold reduction in *hsp16-48* transcription relative to that of the NPT II gene (Table 1). The large decrease in *hsp16-1* transcription was associated with a decrease in the number of adjacent HSEs from two to one, while the decrease in *hsp16-48* transcription, albeit somewhat less than for *hsp16-1*, was associated with an increase in the number of adjacent HSEs from two to three.

The single base deletion in the alternating purine-pyrimidine sequence in the intergenic region had no significant effect on the transcriptional activity of either *hsp16* gene relative to the NPT II gene (Table 1).

All mutations in the *hsp16* gene pair had equivalent effects on both heat shock- and arsenite-induced transcription (Fig. 5), suggesting that the two stimuli act on the *hsp16* promoters through exactly the same sequence elements.

## DISCUSSION

**Conservation of HSP gene promoter elements.** The 5′ flanking DNA of *C. elegans hsp16* genes has the features of an archetypical heat-inducible promoter. Each gene has a 5′ TATA element and a pair of upstream HSEs that closely match the HSE consensus sequence. The HSEs in each pair overlap by 4 bp, which is common to almost all overlapping HSE clusters (e.g., *Drosophila hsp83*, *hsp68*, *hsp23* and *hsp22*, human *hsp70*, *Dictyostelium* DIRS-1 heat-inducible promoter, and soybean small HSP genes [9, 19, 20, 49, 50, 55]). The strict conservation of the HSE between very distantly related organisms (e.g., soybeans and humans) is presumably due to a high sequence specificity of the transcription factors that interact with it and a low probability of compatible mutations in those factors and in all of the essential HSEs in a genome. Whatever the cause, restricted evolutionary divergence in HSP gene promoter sequences enables efficient and tightly regulated transcription of the *C. elegans hsp16* genes in mouse fibroblasts.

Both of the transfected *hsp16* genes were strongly induced by a 42.5°C heat shock or arsenite, which are stimuli that induce the heat shock response in rodent fibroblasts (23). The genes used the same transcription initiation site in *C. elegans* and mouse cells. Under optimal heat shock conditions (2 h at 42.5°C), the *hsp16-1* transcripts accumulated to an estimated 1.2% of mRNA, or 12,000 transcripts per cell, in a mouse cell line that had 12 copies of the gene. This is comparable to the *hsp16-1* transcript abundance of 0.3% of mRNA in optimally induced (4 h at 35°C) *C. elegans*, which has 4 genes per diploid cell (43). After 2 h of heat shock, the *hsp16-1* transcript levels were more than 100 times higher than those of the constitutively expressed NPT II gene.

Efficient expression of the *hsp16* genes was neither dependent on nor noticeably affected by the presence of the BPV upstream enhancer element. Thus, the strength of the *hsp16* promoters appears to be an autonomous property of the *C. elegans* DNA.

Prior to this study, the transcriptional efficiencies of exogenous HSP genes in mammalian cells were uncertain, due to limitations in the transfection methods that were employed. An indeterminate but large proportion of the *Drosophila* HSP genes introduced into monkey COS cells on simian virus 40 vectors (4, 33, 37) or stably integrated into mouse or rat cell chromosomes (8, 13) were transcriptionally repressed, and so the low number of transcripts per transfected HSP gene in these cells would not be an accurate measure of optimal promoter strength. The high transcriptional efficiency of the *C. elegans hsp16* genes that was observed in mouse cells transfected with PN vectors demonstrates that the efficiency as well as the inducibility of HSP gene promoters can be faithfully reproduced in distantly related host cells.

What promoter elements of the *C. elegans hsp16* genes are functioning in mouse cells? The 120 bp of DNA between the HSEs of *hsp16-1* and *hsp16-48* are 78% conserved in the structurally similar *hsp16-2-hsp16-41* gene pair (D. Jones, R. H. Russnak, R. J. Kay, and E. P. M. Candido, J. Biol. Chem., in press). Within this region, both gene pairs have a segment of alternating purines and pyrimidines. Various lengths of alternating purine-pyrimidine sequence are found in the promoter regions of the *Drosophila hsp68*, *hsp27*, and *hsp23* genes (19, 20, 50), a human metallothionein gene (22),

the rat and human somatostatin genes (18), and in many viral enhancers (35). A single base deletion in the intergenic region of the *hsp16-1-hsp16-48* gene pair, which reduced the length of the alternating purine-pyrimidine sequence from 10 to 7 bp, had no significant effect on the function of the promoters in mouse cells. Removing three of the four HSEs and all the intervening upstream sequences from the *hsp16* gene pair reduced the transcriptional activity of both genes, but the loss of these sequences could be fully compensated for by reinserting three more HSEs in a single cluster. The sequences between the HSEs of the *hsp16* genes were not proven to be nonfunctional as promoter elements by these results. They may be responsive to inducers other than high temperature or arsenite. They may enhance promoter activity in the WT gene pair, with their absence in the NF gene pair being offset by a strong promoter resulting from the overlap of four HSEs. Finally, and more probably, they may regulate *hsp16* gene transcription in *C. elegans*, but through sequences and transcription factors that are not present or sufficiently conserved in the mouse.

Some elements of the *hsp16* genes must function abnormally in mouse cells because the ratio of *hsp16-48* to *hsp16-1* transcripts was fivefold lower in mouse cells than in *C. elegans*. While this may have been due to differences in promoter strengths, it is equally possible that the transcripts had disproportionate stabilities in the two organisms. While the *C. elegans hsp16* promoters functioned efficiently in mouse cells, the sequences involved in *hsp16* transcript processing were partially or completely defective despite homology to their equivalents in mammalian genes. Most of the transcripts of both *hsp16* genes were either unspliced or incorrectly processed at their 3′ ends (R. Kay, R. Russnak, and E. P. M. Candido, manuscript in preparation). These defects in pre-mRNA processing may have affected the stability of the *hsp16* transcripts in mouse cells.

**Effect of HSE multiplicity on promoter strength.** In addition to defining the elements of the *C. elegans hsp16* genes that are functional in a distantly related host, we used the wild-type and mutated *hsp16* gene pairs to examine the role of HSEs in controlling transcription in mouse cells. With the MX gene pair, in which the two TATA elements are preserved but all HSEs are deleted, neither gene was transcribed in induced or uninduced cells. Placing a single HSE between the TATA elements (DX) resulted in heat-inducible transcription of the two genes from the correct initiation sites, but transcript levels were significantly lower than those obtained with the WT gene pair. Expanding the single HSE into a cluster of four overlapping HSEs (NF) increased transcription of both genes to a level greater than that of the WT gene pair. Therefore, while a single HSE upstream from the TATA element is necessary and sufficient for heat or arsenite induction in mouse cells, rates of induced transcription are highly dependent on the number of upstream HSEs.

The *Drosophila hsp70* gene has HSEs located 48, 72, 171, and 241 bp upstream from the transcription initiation site (52). Deleted versions of this gene that have retained only the HSE closest to the initiation site were only weakly induced (16) or noninducible (2) in transformed flies and in cultured *Drosophila* cells, respectively. *hsp70* promoters with only the proximal HSE were also inefficiently transcribed in vitro in the presence of extracts from heat-shocked *Drosophila* cells (52). Similarly, genes with a single synthetic HSE in their promoters were not heat inducible in transformed flies (34). Deleted *Drosophila hsp70* promoters that included both the first and second upstream HSEs were much more efficiently transcribed in vivo and in vitro (2, 16, 52).

The HSEs of the *Drosophila hsp70* gene are the binding sites for a heat shock transcription factor (HSTF), which has cooperative binding kinetics to the two HSEs closest to the initiation site (52). Because HSP gene promoter strength is magnified by HSE multiplication in both *Drosophila* and mouse cells, the mechanism by which transcription factors activate the promoters is probably the same in the two organisms.

The initial experiments that defined the essential promoter elements of HSP genes suggested that multiple HSEs were not important for promoter strength in mammalian cells. *Drosophila hsp70* promoters containing only a single upstream HSE were transcribed just as efficiently in monkey COS cells as promoters that included the additional upstream HSEs (33, 37, 39). These genes were on replicating simian virus 40 vectors that would be present in greater than 10,000 copies per cell (31). It now seems probable that a limited supply of HSE-binding transcription factors in these cells was distributed among a large excess of HSEs, with most of the genes being without any factor and therefore inactive, and the remaining genes having only a single bound HSE conferring weak inducibility.

The *Drosophila hsp83* gene is strongly induced by heat shock but is also transcriptionally active under normal growth conditions (60). It has been suggested that the three overlapping HSEs in its promoter may have a very high affinity for HSTF, resulting in preferential expression of this gene when concentrations of active HSTF are low (59), as they apparently are in non-heat-shocked *Drosophila* cells (36). The NF gene pair has a similar cluster of overlapping HSEs and is strongly induced by heat shock in mouse cells, but unlike *hsp83* in *Drosophila*, it is completely inactive under normal growth conditions. There may not be any active heat shock transcription factor in unstressed C127 cells, in which case the endogenous *hsp70* genes that are constitutively expressed in these cells (D. Dixon, personal communication) must be induced by a different mechanism than that proposed for the *Drosophila hsp83* gene.

**Effects of HSE configuration and orientation.** The number of upstream HSEs does not rigorously determine the promoter strength of HSP genes in mouse cells. In the IX gene pair the *hsp16-1* gene lost one of its two HSEs, and its transcript levels dropped 10-fold relative to the WT gene pair levels. The *hsp16-48* gene, on the other hand, exchanged its distal HSE for both the proximal and distal HSEs of *hsp16-1* in this construction, leaving it with three overlapping HSEs, but the IX *hsp16-48* transcript levels were also reduced, twofold relative to the WT gene pair. The new HSEs of the IX *hsp16-48* gene should be functional and properly oriented because they were able to activate *hsp16-1* transcription in the WT gene pair. The low efficiency of the IX *hsp16-48* promoter may be due to some form of HSE incompatibility, such as a nonproductive order of cooperative transcription factor binding. For example, it may be necessary to have the HSE with the highest factor affinity adjacent to the initiation site, as in the *Drosophila hsp70* gene (52).

A single HSE (DX) or an HSE cluster (NF) can activate bidirectional transcription of the *hsp16* gene pair when it is flanked on either side by a TATA element and initiation site. The DX and NF gene pairs have different ratios of *hsp16-1* to *hsp16-48* transcripts, and therefore, in at least one of these cases, the HSEs must have a directional bias independent of downstream sequences. The HSE consensus sequence is formally the same on both DNA strands, so an HSE would

be expected to function in either orientation. However, the sequences of actual HSEs are not the same on both strands, so their orientation could affect promoter strength. If the presumed transcription factors are asymmetric, their probable orientation and effect on transcription could be dependent on the binding strengths of the two alternative positions on the HSE. The orientations of the HSEs of the wild-type *hsp16* gene pair may be optimized for transcriptional efficiency; *hsp16-48* was more strongly induced than *hsp16-1* in the DX gene pair, in which the single HSE is in the natural orientation relative to *hsp16-48* but is in a reversed orientation relative to *hsp16-1*.

While the HSEs are capable of functioning in either orientation, no transcription initiation occurred on the upstream side of the WT HSEs. Therefore, additional sequences are required to initiate transcription. These sequences do not have to include a TATA element because this is absent in a functional human *hsp70* promoter (55).

**Remote HSEs.** The *C. elegans hsp16-48* gene has a 7 of 8 match to the HSE consensus sequence (CTCTAAACTTCAAG) immediately upstream from the coding region and 40 bp downstream from the initiation site (43). This downstream HSE of *hsp16-48* is preserved in the MX *hsp16* gene pair, but cannot activate transcription in mouse cells in the absence of the HSEs immediately upstream from the initiation sites of the two *hsp16* genes. The *Drosophila hsp83* gene has two downstream HSEs located within an intron (17). It is not known whether these HSEs have any effect on heat-induced transcription of this gene. HSEs located 171 and 242 bp upstream from the initiation site of the *Drosophila hsp70* gene bind HSTF and slightly increase promoter strength in vitro (52), and in the case of the *Drosophila hsp26* gene, HSEs located more than 250 bp upstream from the initiation site are essential for high levels of transcription in vitro (11). HSEs that are remote from the initiation site may indirectly increase promoter strength by localizing transcription factors in the vicinity of the HSEs that are directly involved in transcription initiation.

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