Short Communication

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Murine 86-kDa heat shock protein gene and promoter

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Abstract The class of 90 kDa heat shock proteins (Hsp90) is among the most abundant heat shock proteins (Hsps) in eukaryotic cells. In vertebrates, Hsp90 is encoded by two distinct gene families giving rise to products of 84 and 86 kDa. In mice the expression of these two genes, *hsp84* and *hsp86*, vary with respect to each other in responses to stress, and also in response to signals for growth and development. Therefore, as a step towards understanding the molecular basis for the differential regulation of these two genes, we have isolated and characterized genomic clones of the murine *hsp86* gene and its 5′ flanking region. The gene is composed of eleven exons interrupted by 10 introns. The 5′ region contains consensus TATA, several stimulatory protein-1 binding site (SP1) elements as well as six consensus heat shock elements (HSE) 5′ of the transcription start site. An 806 bp fragment of the 5′ promoter region conferred constitutive expression upon a reporter gene and this expression was increased upon heat shock.

INTRODUCTION

The 90 kDa heat shock protein (Hsp90), a member of the stress inducible heat shock protein (Hsp) family is among the most abundantly expressed Hsps found in all cells under normal physiological conditions (Lindquist and Craig 1988; Welch 1992). Genetic studies which show Hsp90 to be essential for cell viability under normal growth conditions (Borkovich et al 1989) are consistent with Hsp90 having important roles in normal cellular functions as well as stress tolerance. Indeed, a growing body of evidence shows that Hsp90 interacts with many cellular factors including calmodulin, actin, tubulin, several kinases and steroid receptors (Parsell and Lindquist 1994; Pratt and Welsh 1994; Xu and Lindquist 1993; Pongratz et al 1992; Schlesinger 1990; Zeimiecki et al 1986; Catelli et al 1985).

In higher eukaryotes, the products of two related genes comprise the Hsp90 class of heat shock proteins. In mice, one gene, *hsp86,* encodes an Hsp of 86 kDa while the product of the second gene, *hsp84* , is 84 kDa. While both the murine *hsp84* and *hsp86* genes are expressed under normal growth conditions, their levels vary with tissue and cell type (Morange et al 1984; Gruppi et al 1991, 1993; Vamvakopoulos 1993). In addition, the expression of *hsp86* relative to *hsp84* is also modulated in response to cellular and environmental stimuli. For example, the constitutive level of Hsp84 at normal physiological temperatures is two-fold higher than Hsp86 (Barnier et al 1987; Ullrich et al 1989; Shyamala 1993) and while both genes are heat shock inducible, *hsp86* displays a higher level of induction than *hsp84* (Barnier et al 1987; Ullrich et al 1989). *hsp86* also shows a greater induction in response to the steroid hormone estrogen in its target tissues (Shyamala 1993). In addition, the level of Hsp86 and not Hsp84 decreases upon cellular differentiation (Barnier et al 1987; Yufu et al 1988). While all these observations confirm that the regulation of the *hsp86* gene is distinct from that of *hsp84,* little is known about the molecular mechanisms which provide for this expression. In order to examine the complex regulation of hsp86 synthesis, we have cloned and determined the structure of the murine *hsp86* gene and its 5′ flanking region.

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Fig. 1 Organization of the *hsp86* gene and genomic clones. A mouse Balb/c genomic library (Clonetech, ML1030j) was screened by plaque hybridization with ^{32}P end-labeled 70 nt probe (5′-TAATGCCTACAGTTTCAAATAATCATTTCTTTCTGGTAAGTTCTTCTATGGCCTAAGCCA-TCACTTAGTT-3′) complementary to sequences in a distal intron (#9) of *hsp86* (Moore et al 1989). To obtain clones carrying the 5′ portion, a mouse P1 library (Genomesystems) was probed by PCR with a primer set specific to the third intron of *hsp86*. Putative clones were confirmed and characterized by southern blot hybridization with a ^{32}P end-labeled 27 nt probe complementary to the 5′ untranslated region of *hsp86* cDNA and ^{32}P labeled cDNA fragments labeled by the random primer method. (A) The *hsp86* gene is shown with exons as boxes and introns (numbered) as lines between them. The 5′ untranslated leader (5′ UTL) and ATG start codon are also indicated. The λ genomic clone λ86–27 is shown with its estimated size and the P1 phage clone P1-86-1 is shown with estimated extent only on the 5′ side of intron 3. Also shown are the fragments subcloned into plasmid vectors to yield p86-3 and p86-1. Restriction sites indicated as E, *EcoRV*, and B, *BamHI*. (B) Derivative of p86-3 which has the *hsp86* 5′ and UTL regions fused to the luciferase reporter gene. The arrow and +1 denote the 5′ end of the *hsp86* mRNA.

RESULTS AND DISCUSSION

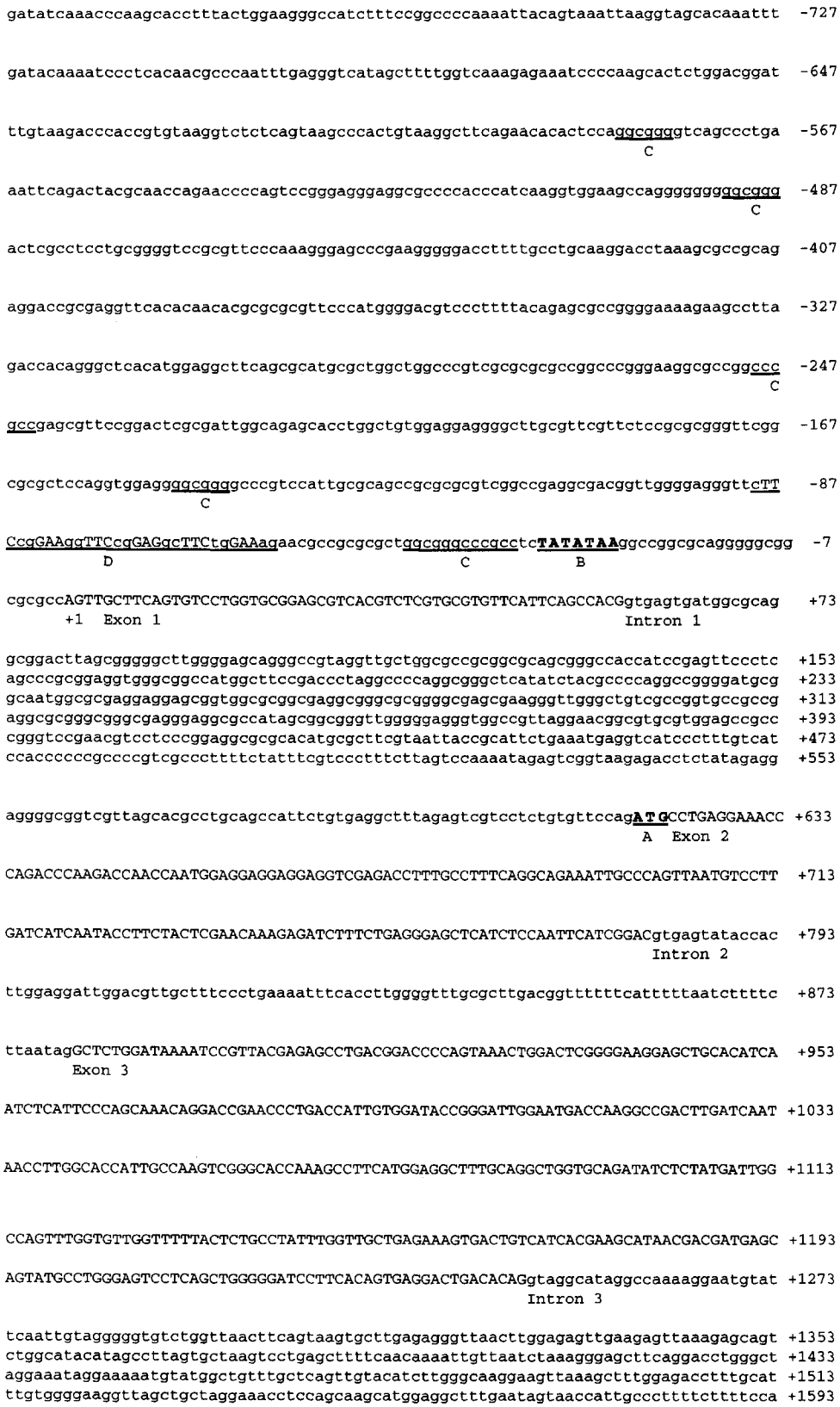
Isolation of murine hsp86 genomic clones

Approximately 2 × 10^6 clones of a murine λ genomic library were screened using an oligonucleotide probe to sequences in previously described intron 9 of murine *hsp86* (Moore et al 1989) as a means to eliminate the isolation of clones carrying pseudogenes. Of the clones isolated from this library, λ86-27 extended from within exon 3 through the 3′ terminus of the *hsp86* gene (Fig.1). To obtain clones which carry the 5′ portion of the *hsp86* gene, a P1 library was also screened using PCR and two *hsp86* intron 3 specific primers as determined by partial sequence analysis of the λ86-27 clone described above. Two P1 clones thus obtained were purified and characterized and one clone (P1-86-1; Fig. 1) was found to contain a minimum of exons 1 through 3 in addition to >10 kb of sequences 5′ of the gene (Fig. 1). The extent of the P1 clone beyond exon 3 was not determined. The combination of these clones yields the entire *hsp86* gene and a significant extent of 5′ sequences.

Organization and nucleotide sequence of the hsp86 gene and 5′ flanking region

Fragments from the λ 86-27 and P1-86-1 library clones (Fig.1) were subcloned into plasmid vectors for DNA sequence analysis and the nucleotide sequence of the *hsp86* introns were determined along with portions of the flanking coding regions which proved consistent with the cDNA sequence (Moore et al 1989). The murine *hsp86* gene, shown in Fig. 2, consists of 11 exons which range in size from 58 to 367 bp interrupted by 10 introns with sizes from 100 to 561 bp, a structure which has been conserved between the mouse *hsp86* and human *hsp89α* genes (Hickey et al 1986). The relative position but not the size of each intron is also conserved between the murine and human homologues, including the position of the first intron at the exact boundary between the untranslated leader (exon 1) and the initiation of translation (ATG). This unusual positioning of the first intron is a feature shared by other members of the *hsp90* gene family including murine *hsp84*, human *hsp89α* and *hsp89β*, chicken *hsp90α* and *hsp90β* and drosophila *hsp82* (see Moore et al 1990; Meng et al 1995). Each *hsp86* intron has the gt/ag consensus splice junction and the consensus derived for the *hsp86* splice sites is well within the range of variability observed in mammals from the consensus sequences (Shapiro and Senepathy 1987; Jacob and Gallinaro 1989).

In addition to the structure of the *hsp86* gene, we also determined the sequence of 806 bp of the 5′ flanking region (Fig. 2). This region contains several consensus sequences which are indicative of a promoter region including a putative TATA box and several SP1 consensus elements. A characteristic feature of heat shock promoters is the presence of heat shock transcription factor binding



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gGTGAACCAATGGGTCGTGGAACAAAGGTTATCTTGCATCTGAAAGAAGACCAAACAGAGTATTTGGAGGAAAGGAGAAT +1673
 Exon 4

AAAGGAGATCGTGAAGAAGCATTCTCAGTTCATTGGCTATCCCATTACTCTCTTTgtaagttacctacagggtaaatttc +1753
                                                              Intron 4

atataattgagataactgaggctgtttagagttctgtgaacctggtgttgtactagacagattagcctgtttggaaaggt +1833
ggtatctgaagaggattgtggagaaattagagaaactactgatgagaggcttcttggttgtacacatttctgagatctgt +1913


cctagttgtataaatttataatgaagtgatttgcctttcaagGTGGAGAAGGAACGAGATAAGGAAGTCAGTGATGATGA +1993
                                            Exon 5

GGCTGAAGAAAAGGAAGAGAAAGAGGAAGAGAAAGAAAAAGAAGAAAAGGAGTCTGATGATAAACCTGAAATAGAAGATG +2073


TTGGCTCTGATGAAGAAGAGGAGGAGAAGAAGGATGGTGACAAGAAGAAAAAGAAGAAGATAAAGGAAAAGTACATTGAT +2153


CAAGAAGAACTCAACAAAACAAAGCCGATTTGGACGAGAAATCCTGATGACATCACTAATGAGGAATATGGAGAGTTCTA +2233


CAAGAGCTTAACTAACGATTGGGAAGAACACTTGGCAGTAAAGgtgagtgacttaaaagcaggttttcaggttgactttt +2313
                                            Intron 5

taaacctctatactcacctatagaaaagcttcgaagtgtaacttagcccagtagagaaagccaattcattttgtccatgg +2393


cattaaaaggttgtttcattttattgcagCATTTTTCTGTTGAAGGACAATTAGAATTCCGGGCCCTTCTTTTTGTCCCA +2473
                                Exon 6

AGACGCGCTCCTTTTGATCTGTTTGAAAACAGAAAGAAAAAGAACAACATCAAGTTGTATGTTCGCAGAGTTTTTATCAT +2553


GGATAACTGTGAGGAATTAATCCCTGAGTATCTGAgtaagtagggatgggaagaatcggcactgttgacagacagaaaat +2633
                                    Intron 6

cccattcgttgggtctgtgttggtgggggactgaggatgtaacctagtggagacggggtttgcctaacttgtttgaggct +2713
ctgggctcagtcccttggaaaaaagccaaaaagattccttaggtttcagaatgattaaataggtgtctttgtttttcccc +2793


agATTTCATTAGAGGGGTAGTGGATTATGAGGATCTCCCTCTAAATATTTCCCGTGAAATGCTGCAACAAAGTAAAATTC +2873
  Exon 7

TGAAAGTTATCAGAAAGAATTTGGTCAAGAAATGCTTAGAACTATTTACTGAACTAGCAGAAGATAAAGAGAACTACAAA +2953


AAGTTTTATGAGCAGTTCTCAAAAAATATAAAGgttggtggtacattttcttgttactggcagttttccaaactaacttg +3033
                                Intron 7

attgggggaggggtcctcacacacttgcttctgcctcccaagtgtggggattaaaggctaatgcttcataaatggctttc +3113


ctgtgcttgtgcttcagCTTGGAATTCACGAGGACTCTCAGAATCGGAAGAAGCTTTCAGAGCTGTTGCGGTACTACACA +3193
                Exon 8

TCTGCTTCTGGGGACGACATGGTTTCTCTGAAGGACTACTGTACCAGAATGAAGGAAAACCAGAAGCACATCTATTTTAT +3273

CACAGgtaatggtcaagtaaacaggtgttttctctacctgttggacagcacttaaggaggcaagaatgtccagcctgtac +3353
     Intron 8

AAGGACCAGGTTGCTAACTCCGCCTTTGTGGAACGTCTCCGAAAGCATGGCTTAGAAGTAATTTATATGATTGAGCCCAT +3513
Exon 9

TGATGAGTATTGTGTGCAACAGCTGAGGGAATTTGAGGGCAAGACCTTGGTGTCTGTTACCAAAGAAGGACTGGAACTTC +3593


CAGAAGATGAAGAGGAAAAGAAGAAACAGGAAGAGAAAAAGACAAAATTTGAGAACCTCTGCAAAATTATGAAAGATATT +3673


TTGGAGAAGAAGGTTGAAAAGgtatgtgaatggttacacatcactcgtagtcagtttggtccttttatctgagttttgac +3753
                        Intron 9

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ttttgttttaattttagGTGGTTGTGTCAAACCGACTGGTGACATCCCCGTGCTGTATTGTCACAAGCACATATGGGTGG +3913
                 Exon  10

ACAGCAAACATGGAGAGAATCATGAAAGCTCAAGCCCTCAGAGACAACTCAACAATGGGTTACATGGCAGCAAAGAAACA +3993


CCTGGAGATAAATCCTGATCACTCCATTATTGAAACCTTAAGGCAAAAGGCAGAGGCTGACAAGAATGACAAATCTGTGA +4073


AGGATCTGGTCATCTTGCTGTATGAAACTGCACTCCTATCTTCTGGCTTCAGTCTCGAAGATCCCCAGACCCATGCTAAC +4153


AGGATCTACAGGATGATCAAGCTTGGTCTAGgtaaattacaggctacctgtaactaacataagggaaagaaacctgttgc +4233
                               Intron 10

aactccccctttaatcctagtgctggggtcagggtgtaaagggacagataggccctttgagttttaggccagtgtggctac +4313
agagttccaggaaaaccaaagctatatatactaaccctggctttaaaaacaaaaaaacgaagttgacttttgtagctatt +4393
gaaacaattggattattgctgttatggaagcctcagctaaagggtttgaatgaaatgaacagtgacctttgtgtttgagg +4473
atggctttgggctggttctgcctctgtcccagtgctggggtttataggcacatacttggcttgccccctatgaataaagt +4553
ttcctttcaggggattggatggggtgttaatgtctaaagcaagtctccgcctccctttagcggacattaactgtaacattt +4633


atttccaaaacaagGTATTGATGAGGATGATCCTACTGTGCATGACACCAGTGCTGCTGTAACTGAAGAAATGCCTCCCC +4713
              Exon  11

TGGAAGGAGATGACGACACATCACGCATGGAAGAAGTAGACTAA                                  +4793
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Fig. 2 Nucleotide sequence of the *hsp86* gene A ~3.6 kb BamHI/EcoRI fragment of the λ86-27 clone and a ~3.0 kb BamHI/EcoRI fragment of the P1-86 clone (Fig.1) were inserted into pUC19 for DNA sequence analysis using the chain termination method (with US Biochemical, Sequenase 2.0). Additional subclones were generated as required for DNA sequence analysis using the M13 universal custom primers. The exon/intron boundaries were determined by comparison with the cDNA sequence (Moore et al 1989). The initiation of transcription is defined as +1. Exon 1 (untranslated leader) and all other exons are shown in caps. The methionine initiation codon (A) and the TATA box (B) are shown in bold and underlined. Other putative transcriptional regulatory elements are underlined and represented as follows: (C), Consensus SP1 sites; (D), The HSE elements with the GAA core shown in caps.

sites, HSE (for reviews see: Lis and Wu 1993; Morimoto 1993). The *hsp86* 5′ region has six contiguous 5 bp nGAAn HSE consensus (Xiao and Lis 1991) repeats cTTCcgGAAggTTCcgGAGgcTTCtgGAAa, starting 28 bp upstream of the TATA box; only one of these, nGAGn, varies from the consensus by a single base. The highly heat shock inducible *hsp86* homologues from humans and chickens also have 6 repeats of the 5 bp nGAAn consensus with the human varying from the consensus by a single base and chicken varying by three bases. In contrast to the HSE consensus elements found in the first intron of human, mouse and chicken *hsp90* genes (reviewed in Moore et al 1990), the first intron of the *hsp86* gene does not contain any HSE elements.

We also examined the *hsp86* 5′ region for consensus regulatory elements for Fos/Jun complex (AP1) and for cAMP binding protein (CRE) since these elements were observed in the 5′ region of the murine *hsp84* gene (Dale et al 1996). Neither of these elements are contained in the *hsp86* 5′ flanking region. Thus, the organization of the *hsp86* and *hsp84* promoters are quite distinct as they each contain a different subset of putative regulatory elements.

Mapping of the transcription start site

To identify the initiation site for transcription, we performed a primer extension analysis using an end-labeled 50 base primer whose 3′ terminus is the complement to the last base of the untranslated leader. This primer crosses the intron 1 splice junction and will thus hybridize to spliced *hsp86* mRNA allowing extension to the 5′ end of the mRNA. The location of the initiation site was determined by alignment of the primer extension product with a dideoxy sequence ladder generated using a 50 base primer of genomic sequence with an identical 3′ terminus to that of the primer extension probe allowing sequencing reactions to be performed using the genomic DNA template (Fig. 3). The major primer extension product revealed that the 5′ end of the mRNA was located 24 bp downstream of the TATA box (shown in Fig. 2), and the untranslated leader was 58 bp in length.

The hsp86 gene contains a functional heat shock promoter

The activity of the *hsp86* promoter was examined by placing the entire untranslated leader and 806 bp of 5′ sequences into a luciferase expression vector. This plasmid, p86-320 (shown in Fig.1), when transfected into 3T3 cells, gave an average of 10-fold activity over the promoterless vector (Fig. 4) demonstrating a functional promoter which has basal activity. This construct was also tested under heat shock conditions where luciferase

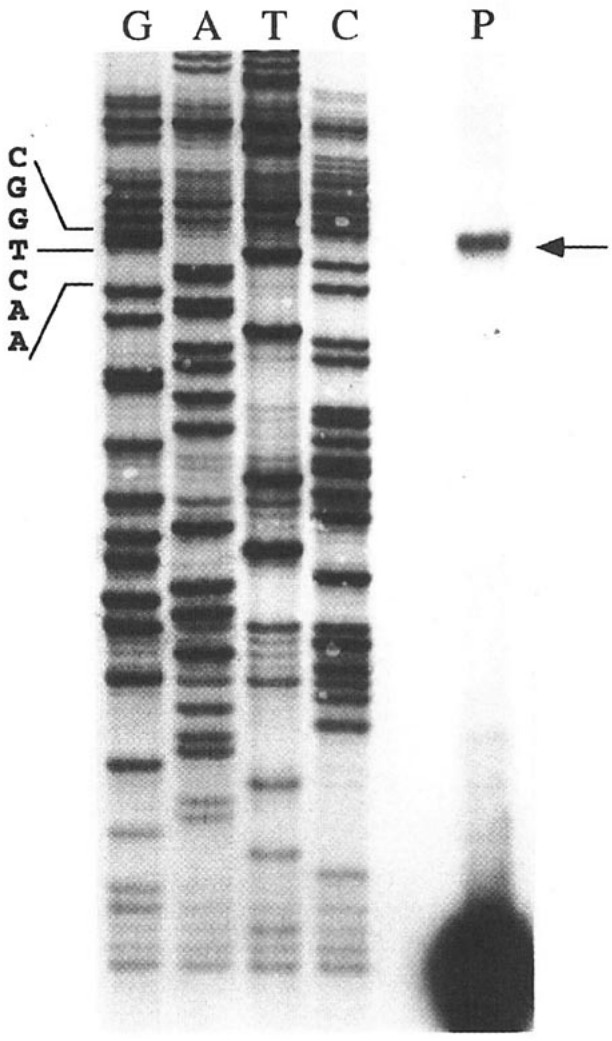

Fig. 3 Mapping of the *hsp86* transcription start site. Primer extension analysis of *hsp86* mRNA was performed using a ^{32}P end-labeled 50 bp primer complementary to the 5′ end of exon #2 of the *hsp86* gene. Primer was annealed to 10 μg total RNA prepared from heat shocked 3T3 cells (see legend of Fig. 4 for details) and extended with reverse transcriptase.Sequencing reactions, loaded in parallel to the primer extension reactions, were performed using the 50 bp primer 86-INT1: ACGGCCCTGCTCCCCAAGCCCC-CGCTAAGTCCGCCTGCGCCATCACTCAC. The major extended product (lane P) is indicated with an arrow. Also shown are dideoxy sequencing reactions and alignment of the sequence with the primer extension product.

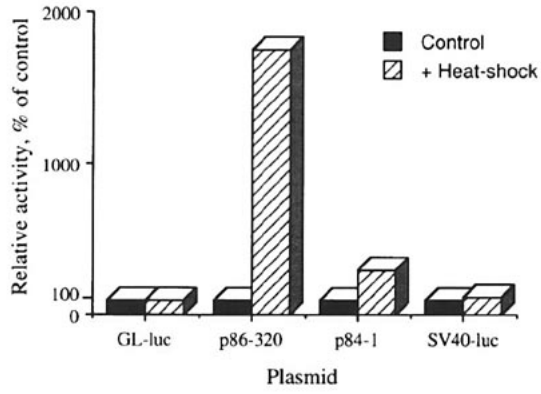

Fig. 4 Functional analysis of the *hsp86* promoter. NIH 3T3 cells were plated at a density of 5 x 10^5 cells/100 mm dish and cultured for 16–18 h. Growth media was then replaced with fresh media for 1 h and transfected with indicated plasmids as previously described (Dale et al 1996). After 4 h of treatment, the media was removed and cells were washed twice in PBS and then cultured in fresh media. At 16 h post-transfection, cells were exposed to heat shock for 20 min by exchanging the growth media with 44°C media and submersion in a circulating 44°C water bath. Cells were then returned to 37°C for 10 h at which time cells were harvested. Control cells were cultured under identical conditions at 37°C. The promoterless pGL2-basic vector (GL-luc) and pGL2-promoter vector (SV40-luc) were purchased from Promega. The luciferase reporter plasmid p86-320 (see Fig.1) was generated by isolating the *Eco*RV and *Bsm*BI fragment of p86-3 (Fig.1) and inserting it into the pGL2-basic vector with the use of linkers and an adapter to preserve the entire untranslated leader sequence. The *hsp84* luciferase plasmid (p84-1) has been previously described by Dale et al 1996. In each experiment transfections were performed in triplicate and data shown are an average of at least three experiments. Luciferase activities measured in cell extracts were normalized to protein (mg/ml) and expressed as percent of the non-heat shocked parallel control. Average values (light units/mg protein) in control non-heat shocked extracts were: GL-luc, 65; p86-320, 5660; p84-1, 1615; SV40-luc, 600.

expression was found to increase by an average of 17-fold above the activity of the non-heat-shock control (Fig. 4).

Recently our laboratory has also cloned and characterized the *hsp84* promoter region (Dale et al 1996) which now allows a comparison of the *hsp86* and *hsp84* promoters. Consistent with the observation that *hsp86* is more heat shock responsive than *hsp84*, the *hsp84* promoter gave only a three-fold induction in luciferase activity (Dale et al 1996). The difference in heat shock inducibility between these two genes may be due to a combination of the size, extent of homology with the consensus, and position of their respective HSE elements. In contrast to the *hsp86*, the promoter region of *hsp84* has only three contiguous 5 bp and among these only two represented the consensus (Dale et al 1996). In addition to the greater number of consensus repeats, the HSEs of the *hsp86* homologues including the mouse are positioned closer to the start of transcription (from –76 to –94) than the HSEs of the *hsp84* homologues (from –440 to –1951). Thus distance from the TATA box may also play a role in the heat shock responsiveness in these genes. Indeed, the chicken *hsp90*β gene which has the HSE homology at the most distal position of –1951 was found to be insensitive to heat shock (Meng et al 1993, 1995). While the pattern of expression from the *hsp86* promoter relative to *hsp84* in the tissue culture assay follows what is observed in vivo, it is interesting to note, however, that the 17-fold induction for *hsp86* is much greater than the 5.5-fold heat shock induction observed for endogenous *hsp86* (Ullrich et al 1989). Thus, the *hsp86* promoter construct

is highly inducible with respect to the endogenous gene which perhaps contains negative regulatory elements which are either not present or not functional on the promoter clone or the endogenous gene may be affected by the surrounding chromatin structure.

ACKNOWLEDGMENTS

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