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# Sequence and Regulation of a Gene Encoding a Human 89-Kilodalton Heat Shock Protein

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**Vertebrate cells synthesize two forms of the 82- to 90-kilodalton heat shock protein that are encoded by distinct gene families. In HeLa cells, both proteins (hsp89α and hsp89β) are abundant under normal growth conditions and are synthesized at increased rates in response to heat stress. Only the larger form, hsp89α, is induced by the adenovirus E1A gene product (M. C. Simon, K. Kitchener, H. T. Kao, E. Hickey, L. Weber, R. Voellmy, N. Heintz, and J. R. Nevins, Mol. Cell. Biol. 7:2884–2890, 1987). We have isolated a human *hsp89α* gene that shows complete sequence identity with heat- and E1A-inducible cDNA used as a hybridization probe. The 5′-flanking region contained overlapping and inverted consensus heat shock control elements that can confer heat-inducible expression on a β-globin reporter gene. The gene contained 10 intervening sequences. The first intron was located adjacent to the translation start codon, an arrangement also found in the *Drosophila hsp82* gene. The spliced mRNA sequence contained a single open reading frame encoding an 84,564-dalton polypeptide showing high homology with the hsp82 to hsp90 proteins of other organisms. The deduced hsp89α protein sequence differed from the human hsp89β sequence reported elsewhere (N. F. Rebbe, J. Ware, R. M. Bertina, P. Modrich, and D. W. Stafford (Gene 53:235–245, 1987) in at least 99 out of the 732 amino acids. Transcription of the *hsp89α* gene was induced by serum during normal cell growth, but expression did not appear to be restricted to a particular stage of the cell cycle. *hsp89α* mRNA was considerably more stable than the mRNA encoding *hsp70*, which can account for the higher constitutive rate of hsp89 synthesis in unstressed cells.**

The 82- to 90-kilodalton (kDa) class of heat shock proteins (HSPs) have long been recognized as cytoplasmic proteins that are abundant in the absence of stress (40, 42, 78) and which are induced to higher levels of synthesis by heat shock. In avian and mammalian cells and tissues, these proteins (hereafter referred to as hsp89) have been found in association with several different regulatory and structural proteins. hsp89 has been shown to interact with several viral oncogene products that possess tyrosine kinase activity, including pp60src (10, 55), and the *yes* (46), *fps* (55), *fes*, and *fgr* (85) gene products. In rabbit reticulocytes, hsp89 has been identified as the 90-kDa component of highly purified preparations of the hemin-controlled translational repressor, an eIF-2α-specific protein kinase (63). hsp89 appears to stimulate the activity of this enzyme. In avian (3, 85) and calf (60) cells, hsp89 has been identified as the non-steroid-binding subunit of the estrogen receptor complex and has since been shown to be a common component of other steroid hormone receptors (33). The steroid-binding component of these receptors appears to be inactive with respect to DNA binding when complexed with hsp89 (30, 58, 66). Specific association of murine hsp89 with tubulin has been reported (67), and calmodulin-sensitive actin binding has been demonstrated in vitro (37, 53). A form of hsp89 is a tumor-specific transplantation antigen in methylcholanthrene-induced tumors in mice (74).

Two forms of hsp89 that differ slightly in molecular mass have been identified in murine (2, 51, 74), human (25), and sea urchin (4) cells. In mouse cells, the proteins are known as hsp84 and hsp86, reflecting their different sizes as determined by gel electrophoresis. Teratocarcinoma cells constitutively express both hsp84 and hsp86 at high levels during proliferation, but upon induction of differentiation, synthesis of hsp86 is specifically down regulated (2). Synthesis of hsp86 and/or hsp84 is induced independently of the other HSPs by estrogen treatment in the murine uterus but not in murine liver or spleen (59). Partial protein sequencing has identified the hsp86 form as the mouse tumor-specific transplantation antigen (74). However, the studies cited above reporting associations between hsp89 and other cellular proteins do not distinguish between the two forms of the HSP.

We have previously isolated several plasmid clones containing inserts homologous to *hsp89* mRNA from cDNA libraries prepared from both heat-shocked (28) and control (65) HeLa cell mRNA. The clones represent two sequence families that do not show cross-hybridization under standard conditions. Two different size classes of *hsp89* mRNA have been demonstrated, which correspond to each family of cDNA (28). The mRNAs are referred to as *hsp89α* (2.95 kilobases) and *hsp89β* (2.7 kilobases) (70). Both mRNAs are coordinately induced by heat shock in HeLa cells (28). The complete sequence of a human *hsp89* cDNA has recently been reported (61). This sequence encodes a protein very similar to mouse hsp84 (51) and corresponds exactly in sequence with the human partial cDNA clone pHS811 (*hsp89β*) isolated by our laboratory (28). This form of *hsp89* mRNA is not induced by the adenovirus E1A gene product, whereas the *hsp89α* form represented by clone pHS801 is strongly induced along with at least one member of the *hsp70* gene family (70). This *hsp70* gene is also induced by serum in the absence of heat shock (80, 81).

In this paper, we report the isolation and characterization of a complete human *hsp89α* structural gene and flanking

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sequences. The transcript from this gene is interrupted by 10 intervening sequences, including one located in the 5′ mRNA leader that is spliced directly at the AUG start codon. The promoter sequence drives heat-inducible expression of a reporter gene, indicating that this *hsp89α* gene encodes a bona fide heat shock protein. The same gene is also expressed constitutively. Quantitative primer extension experiments using a gene-specific synthetic oligonucleotide demonstrated that transcription is enhanced by heat stress and adenovirus infection, while constitutive expression is dependent upon serum. Serum-induced expression of hsp89α is apparently not restricted to a specific stage of the cell cycle in HeLa cells. The spliced *hsp89α* mRNA encodes a protein of 84,564 daltons that differs from the hsp89β sequence (61) in 99 of 732 residues. Comparison of hsp89 protein and derived amino acid sequences from several species suggests that two forms of the gene diverged early during vertebrate evolution and have been conserved. They encode distinct proteins that can be induced independently and may carry out different functions.

## MATERIALS AND METHODS

**Nucleic acid isolation and Southern blot analysis.** High-molecular-weight human DNA was isolated from placenta by the method of Blin and Stafford (7). Total cytoplasmic RNA was isolated from HeLa cells by using a detergent lysis procedure described by Sadis et al. (65). Poly(A)$^+$ RNA was prepared by using standard methods (49). For primer extension experiments, the RNA was precipitated with ethanol an additional time after being adjusted to contain 1 M LiCl. For Southern blot analysis, DNA was digested with restriction enzymes by using conditions recommended by the manufacturer (Boehringer Mannheim Biochemicals). Samples (10 μg) were electrophoresed on 0.7% agarose gels and blotted onto nitrocellulose (72). The blots were hybridized with nick-translated pHS801 or pHS811 plasmid probes as previously described (27, 62) with a final stringent wash for 15 min at 68°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.01% sodium dodecyl sulfate.

**Isolation of the human *hsp89α* gene.** An *Alu*I-*Hae*III human genomic library in phage Charon 4A (44) was screened by hybridization with the *hsp98α*-specific cDNA clone pHS801 (28) under conditions described in detail elsewhere (27). From 1,000,000 plaques, seven hybridizing phage were isolated and plaque purified. DNA was prepared from plate lysates (49) and mapped by restriction digestion and Southern blot analysis with the pHS801 probe.

**Mapping of transcribed sequences and exon-intron boundaries.** Regions of the phage DNA encoding heat-inducible RNA were mapped by differential hybridization of duplicate Southern blots with poly(A)$^+$ RNA isolated either from HeLa cells after 3 h of heat shock at 42°C or from control cells. The RNA was partially degraded with alkali and was $^{32}$P labeled by using polynucleotide kinase (49). *Eco*RI, *Eco*RI-*Bam*HI, and *Hin*dIII fragments (see Fig. 2) were subcloned into M13 phage (50) or Bluescribe KS and SK plasmid vectors (Stratagene). S1 nuclease protection analysis was done with end-labeled double-stranded probes (49) or single-stranded probes obtained from fragments cloned in M13 (12) by using the hybridization procedure described by Favaloro et al. (21). Probes were denatured at 90°C for 10 min and hybridized with 10 μg of total cytoplasmic RNA for 3 h at temperatures between 50 and 60°C, which were determined to be optimal for each probe. The boundaries of the six exons located at the 3′ end of the gene were verified by comparison with overlapping cDNA sequences derived from the cDNA plasmids pHS801 and pHS808 (28) and pCP75 (65), which extend from the poly(A) sequence toward the 5′ end of the gene. The transcription start site and the junction of exons 1 and 2 were determined by directly sequencing poly(A)$^+$ RNA isolated from heat-shocked cells as described below.

**Sequencing.** Restriction fragments cloned into the Bluescript phagemid vectors were used to construct nested deletions with exonuclease III and mung bean nuclease under conditions recommended by Stratagene with the following modification. After the deleted end was made flush by mung bean nuclease digestion, the deleted fragments were excised from the vector by cleavage with *Eco*RI, fractionated on low-melting-temperature agarose gels (54), and subcloned into *Sma*I-*Eco*RI-cleaved M13 vectors for sequencing by the dideoxy-chain termination method (50). This modification avoids the frequent problem of deletion of the sequencing primer site within the phagemid vector. The programs of Schwindiger and Warner (68) were used for sequence collation, and DNA and protein sequence homologies were determined with the PCS program (39). The 5′-untranslated leader sequence of *hsp89α* mRNA was sequenced with reverse transcriptase at 50°C as described by Geliebter (22) by using an end-labeled synthetic oligonucleotide primer with the sequence GTCTGGGTTTCCTCAG GCAT.

**Analysis of *hsp89α* gene expression.** Quantitative primer extension with the end-labeled synthetic oligonucleotide described above was carried out by the same method used for RNA sequencing, except that all four deoxynucleotides were present at 400 μM and dideoxynucleotides were omitted. Primer annealing times was reduced to 15 min, which was found to be optimal in preliminary experiments. This assay gave a linear increase in the primer extension product with inputs of 0.5 to 10 μg of total cytoplasmic RNA. Northern (RNA) blot and slot blot analyses were carried out as described previously (28, 49). Conditions for heat shock at 42°C have also been described previously (29). Serum starvation and stimulation was accomplished by maintaining cells for 48 h in Joklik medium (GIBCO Laboratories) containing 0.5% calf serum (81) and then by suspending them in medium with 10% fresh serum. Cultures were synchronized by double thymidine block as previously described (77). Total cytoplasmic RNA was isolated for analysis at 3-h intervals for 24 h after serum replenishment or refeeding with thymidine-free medium containing 5% fresh serum. Isolation of nuclei and run-on transcription assays were done as described previously (14, 65). RNA from HeLa cells infected with either wild-type adenovirus or the E1A deletion mutant dl312 was generously provided by C. Simon (Rockefeller University, New York, N.Y.) (70). The RNA was isolated 6 h postinfection. The human H4 histone cDNA probe pF0108A was a gift from G. Stein (University of Massachusetts Medical School, Worcester).

**Fusion gene construction and transfection.** A chimeric gene was constructed by standard procedures; the gene contained the 2,100-base-pair (bp) *Nco*I-*Pst*I fragment of the human β-globin (43) gene fused by blunt-end ligation to the 5′ untranslated leader region of the *hsp89α* gene (see Fig. 4). The fusion was made at the filled-in *Ava*II site located 40 bp 3′ from the *cap* site of the *hsp89α* gene. The fusion gene contained the heat shock control element (HSE) and approximately 1,700 bp of additional upstream sequences. The globin gene fragment provided the normal globin initiation codon in the filled *Nco*I site and also contained the entire

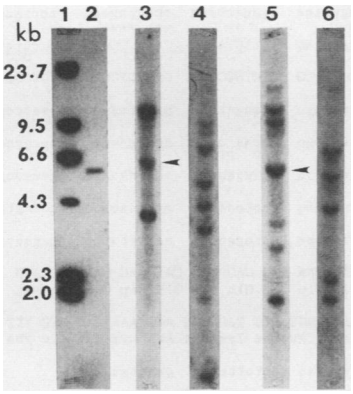


FIG. 1. The genes encoding both forms of human hsp89 are each present in multiple copies. Human DNA was digested with *Bam*HI (lanes 3 and 4) or *Pst*I (lanes 5 and 6). Ten micrograms of each digest was resolved by electrophoresis on a 1% agarose gel and analyzed by Southern blotting. Lane 1, *Hin*dIII-digested lambda DNA. Lane 2 contains an amount of linearized pHS801 (*hsp89α*) plasmid DNA equivalent to a single gene copy per 10 μg of DNA. Lanes 2, 3, and 5 show hybridization with the *hsp89α*-specific probe pHS801. Arrowheads indicate the sizes of fragments expected from the gene cloned in lambda 86. Lanes 4 and 6 have been hybridized with the *hsp89β*-specific probe pHS811.

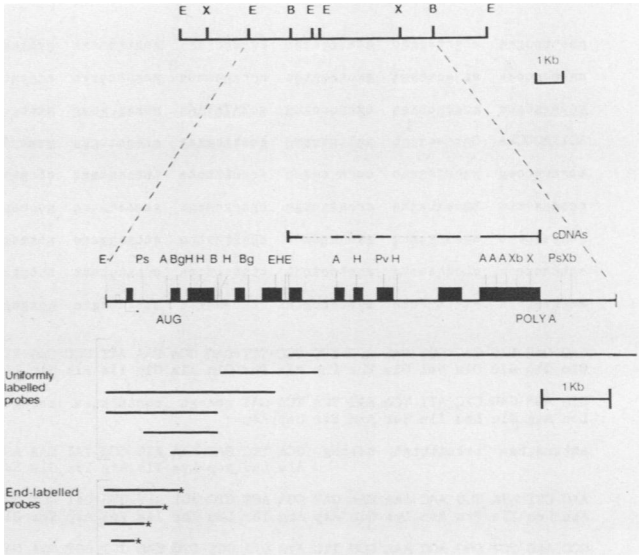


FIG. 2. The structural map of the human *hsp89* gene cloned in phage lambda 86. The restriction map of the genomic fragment cloned in lambda 86 is shown at the top. The expanded section below shows the structure of the region that was sequenced. Exons (■) and the location of the 5′ consensus heat control element (□) are indicated. The arrow marks the transcription start site. The positions of the initiator ATG and the polyadenylation site are also shown. The broken line above the 3′ region indicates the extent of the overlapping cDNA clones that were sequenced to delineate the boundaries of intervening sequences. Indicated under the expanded map are the S1 probes used to define other exons. The precise size and location of the 5′ exon was determined by direct dideoxy sequencing of the mRNA with a synthetic oligonucleotide primer complementary to the coding region at the beginning of exon 2. Restriction sites are abbreviated as follows: A, *Acc*I; B, *Bam*HI; *Bg*, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II; X, *Xho*I; Xb, *Xba*I.

remaining 3′-transcribed sequences as well as about 500 bp of the 3′-flanking sequences. HeLa cells were transfected with CsCl-purified supercoiled plasmid containing the chimeric gene by the calcium phosphate method (24). The cells were put into surface culture at $2 \times 10^6$ cells per 75-cm$^2$ flask in Eagle minimal essential medium with 10% fetal calf serum and were fed again after 16 h of culture. Transfection was carried out by incubating each flask with a precipitate containing 20 μg of the plasmid carrying the chimeric gene and 20 μg of salmon sperm DNA for 16 h. Cells were washed and fed with complete medium as described above and cultured at 37°C. After 30 h, cells were heat shocked at 42°C for 2 h and returned to 37°C for 1 h of recovery. Total cellular RNA was isolated by guanidinium isothiocyanate extraction (49). Samples of RNA (10 μg) were analyzed by quantitative primer extension as described above, with an end-labeled synthetic oligonucleotide specific for the globin mRNA sequence (CAGACTTCTCCTCAGGAGCT).

## RESULTS

***hsp89α* and *hsp89β* mRNAs are encoded by different gene families.** The plasmids pHS801 and pHS811 contain partial cDNA inserts that do not hybridize with each other under standard conditions and specifically identify *hsp89α* and *hsp89β* mRNA, respectively (28). A comparison of the hybridization pattern in Southern blot experiments with digests of genomic DNA shows that each probe hybridized to an entirely different set of restriction fragments (Fig. 1). In addition to fragments corresponding to the *hsp89α* gene subsequently characterized in this report (Fig. 1, arrowheads), there were other hybridizing sequences corresponding to about two to four additional genes or pseudogenes in the human genome. The fragments hybridizing to the *hsp89β* probe indicated that there are also a similar number of copies of this gene sequence.

**Isolation and characterization of a human *hsp89α* gene.** A human genomic library in Charon 4A (44) was screened by hybridization with the *hsp89α*-specific cDNA probe pHS801. Seven positive phage isolates were further analyzed by restriction digestion and Southern blot hybridization with the same probe. Two phage showing the strongest hybridization signals were analyzed by restriction mapping. The two phage were found to contain overlapping segments of the same genomic region (data not shown). One phage, lambda 86, was selected for further structural and nucleotide sequence analysis as described in Materials and Methods. The structural map of lambda 86 is shown in Fig. 2. The boundaries of the region that hybridized with mRNA sequences that increase in abundance after heat shock were localized by differential hybridization. Appropriate restriction fragments were subcloned into M13 and phagemid vectors, and the region shown expanded below the map of the phage in Fig. 2 was analyzed by S1 nuclease hybridization procedures with the indicated uniformly labeled and end-labeled probes. The complete nucleotide sequence of this 7,394-bp segment was also determined. The gene encoded a 5,998-bp primary transcript which contained 10 intervening sequences. In order to precisely localize each exon within the 3′ region of the gene, the sequences of three overlapping cDNA clones isolated independently from heat-shocked (28) and control (65) HeLa cell cDNA libraries were also determined. All three cDNA inserts showed complete sequence identity with the cloned *hsp89α* gene and permitted the identification of the exons covered by the cDNA regions shown in Fig. 2. We were unable to identify the transcription start site and the 3′ boundary of the small first exon by S1

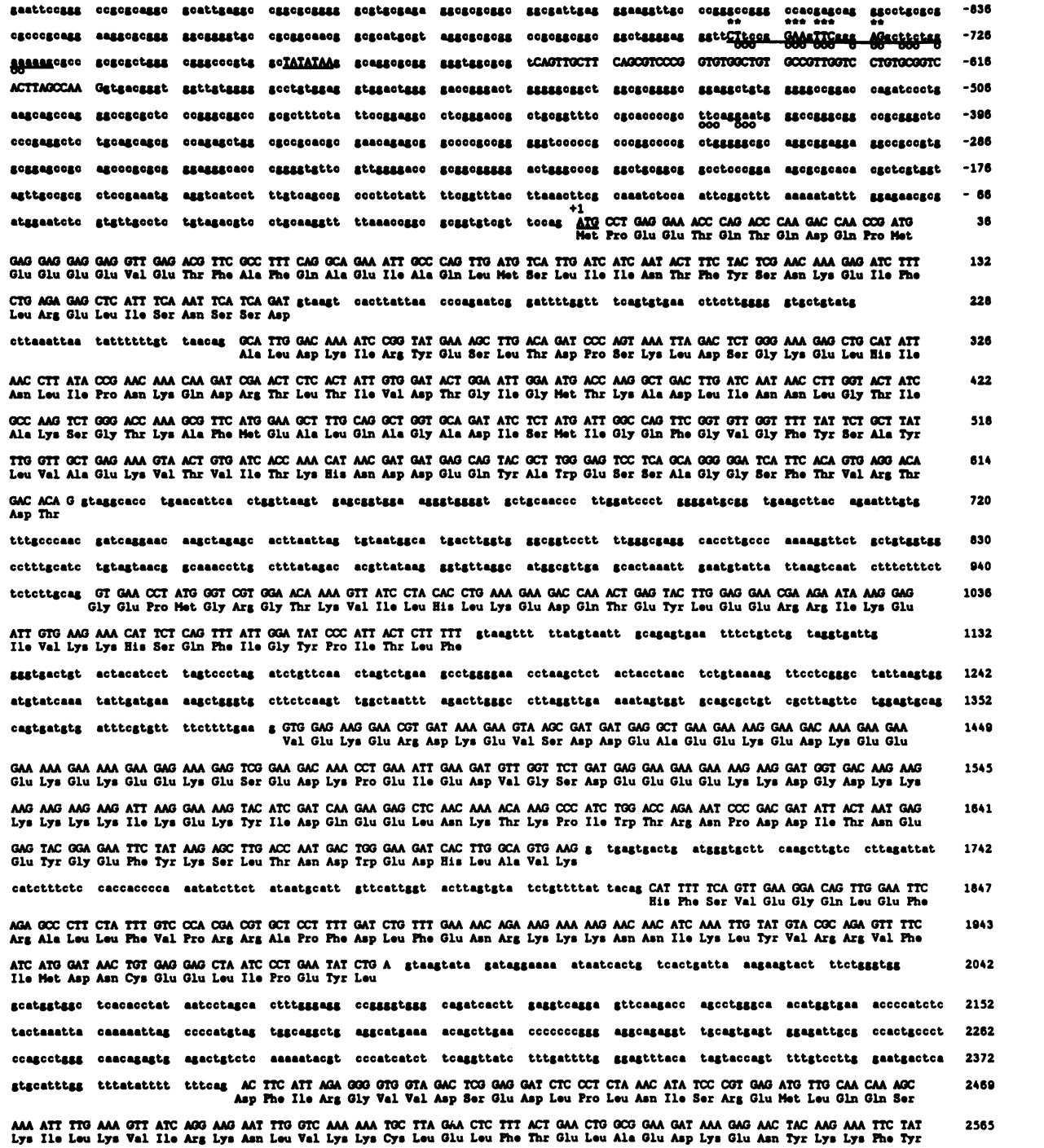

FIG. 3. Nucleotide sequence of the human *hsp89α* gene and deduced amino acid sequence of the polypeptide. The gene contains consensus heat shock control elements (Pelham consensus [56] is in upper case, underlined, and marked by stars; Xiao and Lis consensus [83] is marked with circles below the sequence), and a typical TATA (underlined, upper case). Exons are also shown in upper case; the introns and untranscribed sequences are shown in lower case. The 3′ polyadenylation signal is underlined, and the polyadenylation site is indicated with an underline and a star above. Numbering of nucleotides is from +1 at the ATG.

nuclease methods. This information was obtained by directly sequencing the RNA with a synthetic oligonucleotide primer which had a sequence that terminated at the putative start codon of the protein-coding region. The fact that we were able to obtain a clear sequence with unfractionated poly(A)$^+$ RNA and the sequence identity with three different cDNA clones strongly suggest that the gene cloned in lambda 86 produces the major *hsp89α* transcript in HeLa cells.

**Sequence and organization of the *hsp89α* gene.** The nucleotide sequence of the *hsp89α* gene and surrounding regions and the deduced amino acid sequence of the protein is shown in Fig. 3. The A of the translation start codon is designated

FIG. 3—*Continued*

as +1. Underlined sequences between −740 to −725 in the 5′-flanking region correspond to HSEs. A sequence similar to the HSE described by Pelham (56) is indicated in uppercase letters. Small circles beneath the sequence indicate two perfect and one imperfect repeats of the more simple consensus HSE sequence suggested by Xiao and Lis (83). A single TATA element was found 30 bp downstream from the HSE and 22 bp upstream from the transcription initiation site identified by RNA sequencing. Overlapping the 3′ end of the underlined HSE region was the sequence TGGAAAAAG, which is similar to the serum control element (SRE) identified in a human *hsp70* gene (82). This sequence was located between the HSE and the TATA in both genes. The *hsp89α* promoter contained a GGCGGG sequence corresponding to a putative SP1-binding site located at position −814.

The transcribed region contained 11 exons that ranged in size from 58 to 729 bp. All of the introns were flanked by typical splice consensus sequences that followed the GT-AG rule. The 58-bp exon 1 encoded the entire 5′-untranslated mRNA leader and was separated from exon 2 by the longest

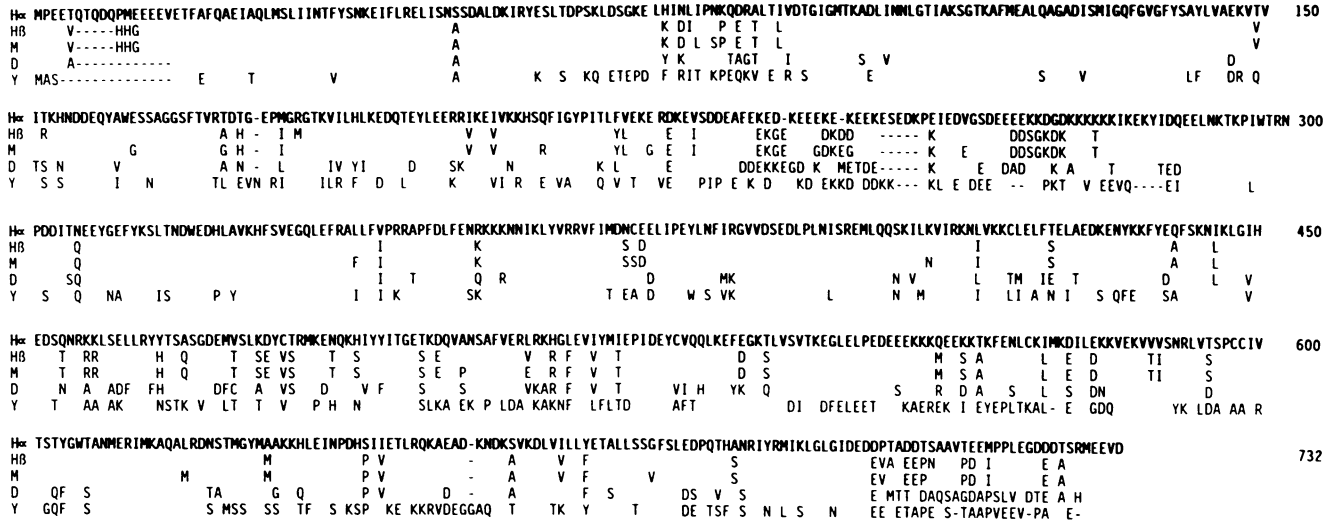

FIG. 4. Comparison of the amino acid sequence of human hsp89α with that of hsp89β and hsp90 proteins from other species. The upper sequence (Hα) is that of the protein derived from the human *hsp89α* gene. Aligned below it are the human hsp89β (Hβ) (61), mouse hsp84 (M) (51), *Drosophila* (D) (6), and *S. cerevisiae* hsp83 (Y) (20) protein sequences. Blank spaces indicate identity with the human hsp89α sequence, letters indicate amino acid substitutions, and dashes represent gaps introduced to maximize alignment.

intron of the gene (572 bp). The splice junction was unusual in that it was immediately adjacent to the initiator ATG. Excision of the first intron would create the sequence CCAA GATG, which is similar to the CCA/GCCATG sequence determined by Kozak (38) to be important for translation in eucaryotes. The adenosine at position −3 is thought to be particularly important for high translational efficiency (38). Excision of the single intron of the *Drosophila hsp82* gene also would bring an adenosine into the −3 position relative to the ATG. An additional feature common to both the human and *Drosophila* genes is the presence of one perfect and several imperfect Xiao and Lis HSE sequences (83) located within intron 1 (indicated by circles under the sequence in Fig. 3). The 5′-flanking region and the first intron are the only locations within the 7,324-bp fragment containing the human *hsp89α* gene in which the TTCNNGAA sequence was found.

The rather long 3′-untranslated region of *hsp89α* mRNA contained 663 nucleotides and was AT rich (63% overall). The 100 nucleotides immediately after the stop codon contained over 70% A+T. There was an atypical polyadenylation signal, AACAAA (underlined in Fig. 3), located 17 nucleotides upstream from the polyadenylation site. The cDNA clone pHS801 was identical in sequence to the 3′ end of the gene, including the AACAAA sequence, and terminated with a poly(A) stretch of over 20 nucleotides. This identified the site of polyadenylation indicated in Fig. 3 with a star and an underline.

**Human hsp89α protein sequence.** The deduced amino acid sequence contained 732 residues with a predicted unmodified molecular mass of 84,564. As is the case for the other heat shock proteins, the hsp89 sequence has been highly conserved evolutionarily. A comparison of the human hsp89α and hsp89β protein sequences with mouse hsp84, *Drosophila* hsp82, and *Saccharomyces cerevisiae* hsp90 is shown in Fig. 4. The amino acid sequence of hsp89α differs at 99 residues from the hsp89β sequence. A major difference between the two human proteins is the presence of an additional block of 5 amino acids (QTQDQ), located near the amino terminus of hsp89α, that was absent in hsp89β. The human hsp89β protein differs from mouse hsp84, which also lacks the QTQDQ sequence, at only 22 residues. Most of the substitutions that differentiate the hsp84-hsp89β proteins are conservative. Thus, the human hsp89β protein is clearly more similar to the mouse hsp84 sequence that to human hsp89α. The carboxy-terminal sequence, EEVD, is conserved not only among the hsp89 proteins of several species (6, 20, 51, 61) but is also found at the carboxy terminus of hsp70 (5, 23, 31, 32, 45, 47, 48) and the hsp70 cognate (19, 71). Human hsp89α protein shows 76% amino acid sequence homology with *Drosophila* hsp82 and 58% homology with the heat-inducible form of *S. cerevisiae* hsp90 (20). The three vertebrate hsp89 proteins have 7 to 12 additional amino acid residues at the amino terminus that are absent in the *Drosophila* and *S. cerevisiae* proteins. Frequent stretches of interspersed acidic and basic amino acids are characteristic of all the hsp89 proteins. The longest region in hsp89α falls between residues 222 and 294. Despite the differences in amino acid sequence, hydropathy plots (data not shown) of the human hsp89α and hsp89β proteins are virtually identical, which would indicate a high degree of structural similarity.

**The *hsp89α* gene contains a functional heat shock promoter.** In order to test whether the gene we isolated encodes a heat-inducible form of hsp89 or a constitutively expressed heat shock cognate protein, we constructed a β-globin fusion gene containing the hsp89 promoter region which is depicted in Fig. 5A. Figure 5B shows the response of this gene to heat stress after transfection into HeLa cells. Transfected and untransfected cells were brought to 42°C for 2 h to initiate heat shock gene transcription and then returned to 37°C for 1 h to allow accumulation of spliced mRNA transcripts (65, 84). RNA was prepared and assayed by quantitative primer extension with a globin mRNA-specific oligonucleotide. Induction of *hsp89α* mRNA was also measured with the specific oligonucleotide used to determine the 5′ leader sequence of the mRNA. In Fig. 5B it can be seen that no transcripts were detected with the globin-specific probe in untransfected cells (lanes 1 and 2). A low level of a transcript giving the expected 88-nucleotide primer extension product was present in cells transfected with the fusion gene at 37°C (lane 3), and the amount of this transcript increased by

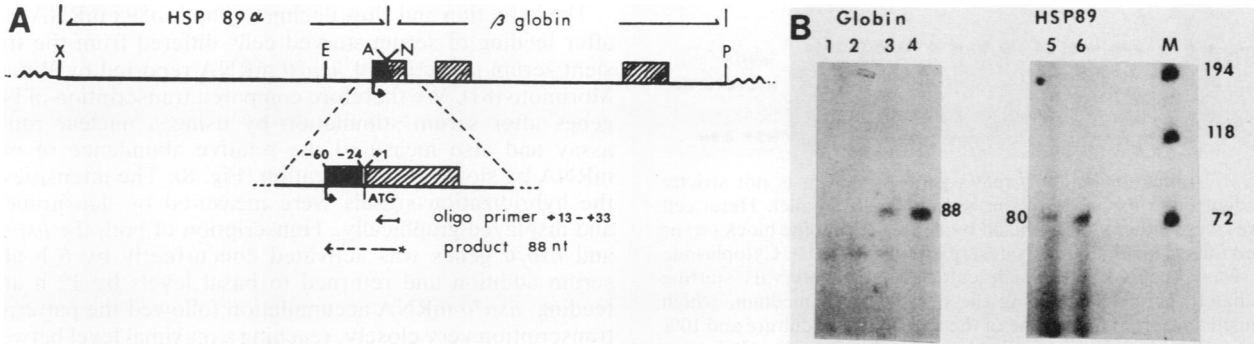


FIG. 5. The promoter of the cloned *hsp89α* gene can drive heat-induced transcription of a globin reporter gene. (A) Structure of the chimeric *hsp89α*-β-globin gene. Construction of the fusion gene is described in Materials and Methods. Restriction sites are abbreviated as follows: X, *Xho*I; E, *Eco*RI; A, *Ava*II; N, *Nco*I; P, *Pst*I. Wavy lines indicate vector sequences; solid lines are the inserted fragments. Transcribed sequences of the *hsp89α* gene (■) and the exons of the globin gene (▨) are indicated. The expanded map shows the details of the fusion site, the position of the oligonucleotide primer, and the expected primer extension product from the fusion transcript. (B) Total cytoplasmic RNA was isolated from untransfected HeLa cells (lanes 1 and 2) or cells transfected with the *hsp89α*-β-globin fusion gene shown above (lanes 3 to 6). Cells were incubated at 37°C (lanes 1, 3, and 5) or were heat shocked for 2 h at 42°C and allowed to recover at 37°C for an additional hour (lanes 2, 4, and 6). Samples (3.6 μg) of RNA were analyzed by primer extension with end-labeled synthetic oligonucleotides complementary to either the globin transcript (lanes 1 to 4), or specific for *hsp89α* (lanes 5 and 6). Primer extension products were analyzed by electrophoresis on denaturing polyacrylamide gels and autoradiographed. The sizes in nucleotides of end-labeled *Hae*III fragments of ϕX174 phage DNA (lane M) as well as the primer extension products are indicated.

approximately threefold after heat shock (lane 4). The same RNA preparations from the transfected cells showed similar changes in the level of endogenous *hsp89α* transcripts (lanes 5 and 6). Thus, the transfected globin fusion gene appears to be regulated in parallel with the natural *hsp89α* transcript and is expressed at normal temperature but at a higher level in response to heat stress.

**Induction of the cloned *hsp89α* gene by adenovirus infection and serum.** Transcription of human *hsp89α* but not *hsp89β* is directly or indirectly activated in concert with one form of human *hsp70* by the adenovirus E1A gene product (70). To determine whether the *hsp89α* gene that was isolated is responsive to E1A, we assayed RNA preparations from HeLa cells 6 h after infection with wild-type adenovirus or with the E1A-deficient mutant adenovirus dl312 (Fig. 6A). Lanes 1 and 2 show the primer extension products obtained from RNA isolated from cells that had been incubated at 42°C for 3 h (lane 1) or 5 h (lane 2) with the *hsp89α* gene-specific oligonucleotide primer. In lanes 3 and 4, the levels of *hsp89α* mRNA in parallel cultures infected with wild-type and mutant adenovirus are compared. The level of *hsp89α* transcript in cells infected with the E1A-deficient adenovirus mutant (lane 3) was typical of that found in uninfected cells. The abundance of the *hsp89α* transcript was increased three- to fivefold by infection with wild-type adenovirus (lane 4), indicating that the *hsp89α* gene is responsive to the E1A gene product.

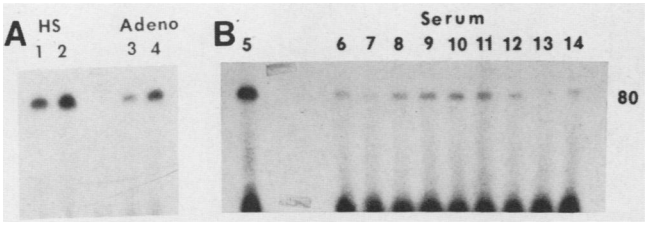


FIG. 6. Induction of the *hsp89α* gene transcript by the adenovirus E1A gene product and by serum. (A) Samples of total cytoplasmic RNA were analyzed by quantitative primer extension with the *hsp89α*-specific oligonucleotide primer as described for Fig. 5. Lanes: 1, 2 μg of RNA from uninfected HeLa cells heat shocked at 42°C for 3 h; 2, 2 μg of RNA from uninfected cells heat shocked at 42°C for 5 h; 3, 5 μg of RNA from cells 6 h after infection with the E1A-deficient adenovirus mutant dl312; 4, 5 μg of RNA from cells 6 h after infection with wild-type adenovirus. Unstressed cells were infected at 37°C. (B) HeLa cells were serum starved and suspended in fresh medium containing 10% fresh serum as described in Materials and Methods. Cytoplasmic RNA was isolated at time zero (lane 6) and at 3-h intervals for a period of 24 h (lanes 7 to 14). Ten micrograms of each RNA sample was analyzed by primer extension as described above. Lane 5 is the primer extension product obtained from 2 μg of RNA from cells incubated for 3 h at 42°C.

The human *hsp70* gene that is activated by the adenovirus E1A product is also regulated by serum (70, 81). Since the *hsp89α* promoter region contains a similar SRE-like sequence, we investigated whether this gene is also serum responsive. HeLa cells were serum starved for 48 h in medium containing 0.5% calf serum and then fed with medium containing 10% serum. The level of the *hsp89α* gene-specific transcripts was then measured by primer extension over the following 24 h (Fig. 6, right panel). Serum-starved cells before refeeding contained a considerably lower amount of *hsp89α* mRNA than was found in exponentially growing cultures (lane 6). After addition of 10% fresh serum, induction of the *hsp89α* transcript was evident beginning at hour 6 (lane 8). The mRNA increased in abundance by approximately fourfold through hours 12 to 15 (lanes 10 and 11) and then declined to near the original level. Other experiments (data not shown) in which fresh serum alone was added to depleted cultures showed a similar induction of *hsp89α*. Feeding serum-depleted cells with fresh medium containing 0.5% serum produced less than a 50% increase in the mRNA. Thus, a serum constituent rather than some other component of the medium controlled the level of *hsp89α* mRNA in unstressed cells.

Serum induction of *hsp89α* in HeLa cells is not restricted to a specific stage of the cell cycle. When deprived of serum, HeLa cells, like many transformed cell lines (18), arrest cell division at random points within the cell cycle. Parallel experiments were conducted in which cells were either starved for serum as described above or synchronized by a double thymidine block. *hsp89α* mRNA levels were then

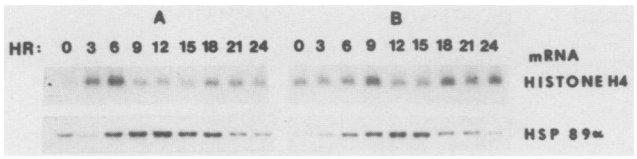


FIG. 7. Induction of the *hsp89α* gene by serum is not strictly dependent upon the stage of the cell cycle. In parallel, HeLa cell cultures were either synchronized by double thymidine block (A) or starved for serum and refed as described for Fig. 6 (B). Cytoplasmic RNA was prepared from each culture at 3-h intervals starting immediately before suspending the cells in fresh medium, which contained 5% serum in the case of the synchronized culture and 10% serum in the case of the serum-depleted cells. Samples (10 μg) of RNA were analyzed for H4 histone and *hsp89α* mRNA levels by Northern blot hybridization with nick-translated pF0108A and pHS801 plasmid DNAs, respectively, as probes.

determined by Northern blot analysis with the pHS801 probe. *hsp89α* mRNA accumulation in synchronized cells released from a double thymidine block (Fig. 7A) was compared with that in asynchronous, serum-stimulated cultures (Fig. 7B). The level of H4 histone mRNA, which is abundant only during S phase (16, 26, 57), was also measured as an indicator of cell synchrony. The cells released from the thymidine block showed a rapid synchronous entry into S phase, as was indicated by accumulation and decline of histone mRNA. The serum-starved cells refed with fresh serum showed little synchrony by this criterion. The temporal pattern and intensity of the increase and decrease of *hsp89α* mRNA, however, was similar in both cultures and did not appear to be related to the stage of the cell cycle. Since the thymidine block was released by suspending the cells in thymidine-free medium containing fresh serum 16 h after the previous feeding, it is likely that the observed stimulation of *hsp89α* mRNA accumulation in this experiment was a serum effect.

The induction and slow decline of the *hsp89α* mRNA level after feeding of serum-starved cells differed from the transient serum induction of *hsp70* mRNA reported by Wu and Morimoto (81). We therefore compared transcription of both genes after serum stimulation by using a nuclear run-on assay and also measured the relative abundance of each mRNA by slot blot hybridization (Fig. 8). The intensities of the hybridization signals were measured by densitometry and displayed graphically. Transcription of both the *hsp89α* and *hsp70* genes was activated concurrently by 6 h after serum addition and returned to basal levels by 12 h after feeding. *hsp70* mRNA accumulation followed the pattern of transcription very closely, reaching a maximal level between 6 and 9 h after feeding and declining rapidly thereafter. In contrast, the levels of *hsp89α* mRNA remained elevated long after the period of highest transcription had passed. *hsp89α* mRNA levels were highest 15 h after feeding and declined much more slowly. Despite the decline in the transcription rate, *hsp89α* mRNA levels remained elevated 24 h after serum addition. This indicated that *hsp89α* mRNA does not share the characteristically short half-life of *hsp70* mRNA (73). The longer half-life of *hsp89α* mRNA appears to be responsible for the higher constitutive synthesis of the protein in unstressed cells.

## DISCUSSION

Three lines of evidence indicated that the gene described in this report encodes the major species of *hsp89α* mRNA expressed in HeLa cells both in response to heat shock and during normal growth conditions. First, the sequences of the 3′ half of the protein-coding region and the entire 622-nucleotide 3′-untranslated region are identical to sequences contained in three independently isolated cDNA clones prepared from RNA isolated from heat-shocked (28) and normally growing cells (65). Also, the gene encoded an mRNA with the same 5′-untranslated leader sequence that

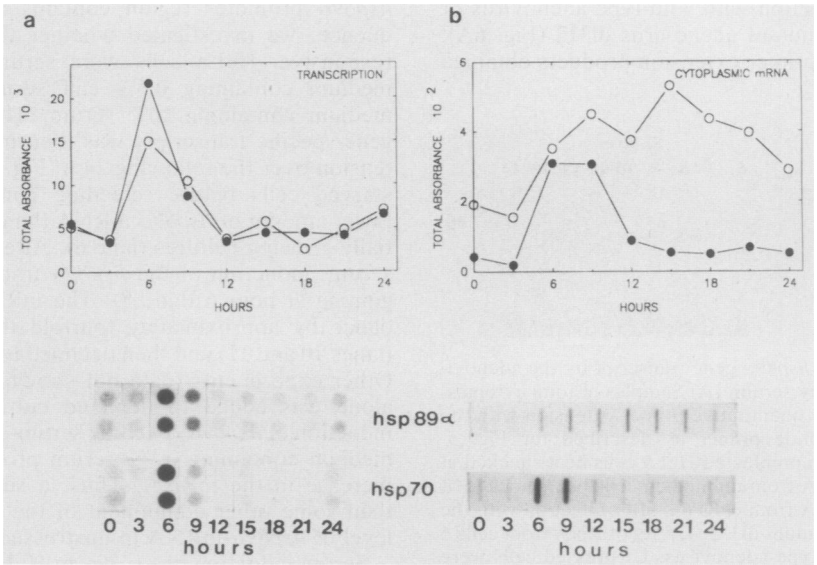


FIG. 8. Transcription and accumulation of *hsp70* and *hsp89α* mRNA after feeding of serum-depleted HeLa cells. Nuclei were prepared at 3-h intervals from the serum-depleted and serum-stimulated HeLa cells described for Fig. 7. (a) Labeled nuclear run-on transcripts were hybridized to filters containing duplicate 10-μg dots of the *hsp70*-specific cDNA plasmid pHS709 (hsp70) (●) or the *hsp89α*-specific cDNA pHS801 (hsp89α) (○). The autoradiograms shown below were analyzed by densitometry, and the results are presented graphically. (b) Samples (5 μg) of cytoplasmic RNA were analyzed by slot blot hybridization with the same *hsp70*-(●) or *hsp89α*-specific (○) cDNA plasmids as probes. The results obtained by autoradiography and densitometry are presented in the graph above the autoradiographed slot blot. For photographic purposes, the *hsp70* slot blot autoradiogram that is depicted was exposed for a longer period of time than the *hsp89α* slot blot.

was obtained by direct RNA sequencing. Finally, primer extension experiments with a synthetic oligonucleotide complementary to the predicted mRNA sequence identified a single RNA species that is both expressed constitutively at 37°C and present at elevated levels in response to heat shock. Other products were occasionally detected when much less stringent conditions were used for primer extension (data not shown). Whether these products represent different *hsp89* mRNAs encoded by the other members of the gene family detected by Southern blot analysis (Fig. 1) is not clear. However, greater than 0.5% of total poly(A)$^+$ mRNA from control cells and greater than 1.5% of total poly(A)$^+$ mRNA from heat-shocked cells can form hybrids with the pHS801 partial cDNA sequence (D. Lloyd, unpublished observation).

The presence of intervening sequences in a heat shock protein gene can no longer be considered an unusual phenomenon in higher organisms. The heat shock genes were first characterized for *S. cerevisiae* and *Drosophila melanogaster*, two organisms with concise genomes which in general contain few interrupted genes. The first heat shock gene shown to contain an intervening sequence was *hsp82* of *D. melanogaster* (6). To date, a human *hsp27* gene (27), the *Caenorhabditis* small heat shock protein genes (64), and a gene for a chicken 108-kilodalton heat shock protein (36) have also been shown to encode spliced mRNA molecules. The chicken ubiquitin gene that is induced by heat shock also contains an intron (9). The absence of intervening sequences may be characteristic only of genes that encode the heat-inducible forms of *hsp70*, which appear to lack introns in all organisms including humans (45).

Despite the presence of intervening sequences, newly synthesized *hsp89α* mRNA accumulates rapidly in the cytoplasm of HeLa cells heat shocked at 42°C, while processing or transport of several non-heat shock mRNAs is inhibited or delayed (65). The intron-exon junctions of the heat shock gene do not appear to be remarkable except in that the splice site sequences match the primate consensus (69) very closely. It is possible that the heat shock gene transcripts may have a kinetic advantage over non-heat shock mRNA precursors in cells undergoing heat stress when RNA-splicing capacity is reduced (8, 34, 84).

The position of the first intron has been conserved in the human *hsp89α* gene and in the *hsp82* gene from four different *Drosophila* species (6). A single exon encoding the entire 5′-untranslated mRNA leader and the unusual location of the splice junction adjacent to the initiator ATG codon is retained in all cases. The nucleotide sequences surrounding both the 5′ and 3′ splice sites are also conserved. The *Drosophila* consensus sequence for the 12 nucleotides surrounding the 3′ terminus of the first exon is ATACAAG/GTRAG. The human sequence differs at only three positions, being AGCCAAG/GTGAC (differences are underlined). The *Drosophila* acceptor splice junction has the less specific consensus YYNTTNCAG/ATG. The equivalent human sequence, TCGTTCCAG/ATG, is completely consistent with the *Drosophila* consensus sequence. The sequences surrounding the ATG that are important for efficient translation are created by the splicing event. Evolutionary pressure for efficient translation of heat shock mRNA during stress may therefore have led to the conservation of sequences at this splice junction. These conserved sequences are not found in the other introns. An additional notable feature of the first intron of the *hsp89α* gene is the presence of HSE sequences that could potentially bind heat shock transcription factor (83). In each of the *hsp82* gene sequences published for the four *Drosophila* species, the intron also contains at least one partial or complete HSE consensus sequence (6). Heat-inducible transcription from the human *hsp89α* promoter does not require the first intron, as evidenced by expression of the *hsp89α*-β-globin gene reported here. Whether the internal HSE-like elements may nonetheless function to enhance transcription of the *hsp89α* gene is currently under investigation. It is possible that the location of the first intron could have been conserved through evolution as a consequence of the presence of HSE elements which may act in concert with those in the 5′-flanking region of the gene.

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Human hsp89α        MPEETQTQDQPMEEEEVETFAFQAE
Mouse hsp86         MPEETQTQDQPMEEEEVETFAFQAE
Calf ERBP           MPEETQA...
Chick hsp90         MPEAVQTQDQPM-EEEVETFAFQAE
Rabbit HCR          MPEEVQTQDQPMETFAVQTFAFQAE
Human hsp89β        MPEEV-----HHGEEEVETFAFQAE
Mouse hsp84         MPEEV-----HHGEEEVETFAFQAE
Drosophila hsp82    MPEEA-----------ETFAFQAE
Yeast hsp83         MAS-------------ETFEFQAE
T. cruzi            M---------------TETFAFQAE
E. coli htpG        MK--GQ----------ETRGFQSE
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FIG. 9. Comparison of amino-terminal hsp89α and hsp89β protein sequences. Sequences of the following hsp89 homologs were taken from the indicated references: mouse hsp86 (74), mouse hsp84 (51), calf ERBP (estrogen receptor binding protein) (60), chick hsp90 (N. Binart, personal communication), rabbit HCR (hemin-controlled repressor) (63), human hsp89β (61), *Drosophila* hsp89 (6), *S. cerevisiae* hsp83 (20), *Trypanosoma cruzi* heat shock protein (17), and *E. coli* htpG gene product (1).

The deduced amino acid sequence of the human hsp89α protein along with the human hsp89β sequence determined by Rebbe et al. (61) allows for the classification of other vertebrate hsp89 homologs as either α-like or β-like. The N-terminal amino acid sequences that have been reported for hsp89 proteins from a variety of organisms are aligned in Fig. 9. The vertebrate hsp89 sequences fall into two clearly delineated classes based on the presence or absence of a glutamine-rich segment (T/VQTQDQ) separating two groups of acidic residues (PEEV/T and M/GEEE[E]V). Human hsp89α contains the glutamine-rich sequence, while human hsp89β does not. Conservation of the distinct amino acid sequences of the α and β forms across phylogenetic divisions is greater than the sequence conservation between the two forms within a single species. The differences between human hsp89α and human hsp89β (99 residues) are greater than the differences between human hsp89β and mouse hsp84 (Fig. 4). hsp84 is 97% identical to human hsp89β, differing at only 22 residues, all of which also differ from the sequence of human hsp89α. Human hsp89α, in contrast, is only 83% homologous with mouse hsp84 but contains the same N-terminal 30 amino acids as mouse hsp86 (74) (Fig. 4). The 200 C-terminal amino acids of mouse hsp86 recently deduced from a cDNA sequence are also identical to those of human hsp89α (V. Legagneux, personal communication). Thus, murine hsp86 is clearly hsp89α, and murine hsp84 is hsp89β. The sequence of the chicken steroid receptor-associated hsp90 that has recently been completed by N. Binart (personal communication) indicates that this protein is an α form and differs from human hsp89α at only 32 residues (96% identity). The N-terminal 7 amino acid residues of the calf steroid receptor-binding protein (60) contain the glutamine-rich sequence, which indicates that this hsp89

is also an α form. The remaining 78 residues that have been sequenced show only four differences from the corresponding regions of human hsp89α but 14 differences from human hsp89β. The rabbit hsp89 protein found in association with the hemin-controlled repressor protein kinase (63) is also an α form.

The functions of the hsp89 proteins are still unclear. hsp89 is an abundant protein that exists primarily as a dimer (37, 78). The two different forms of hsp89 may interact to form a heterodimer. However, differential regulation of the two genes during development (2) would argue that heterodimer assembly, if it occurs, is not obligatory. Most studies have suggested that hsp89 is a soluble protein that is localized within the cytoplasmic compartment (40, 42, 78). Immunofluorescence studies have shown a general distribution of hsp89 throughout the cytoplasm of murine and chicken cells (75) with particular accumulation in ruffled borders (37) and in regions containing microtubules (67). In *D. melanogaster*, hsp82 becomes more localized toward the periphery of the cell at elevated temperatures (13). One report which used immunogold labeling suggested that small amounts of hsp84 enter the nucleus of mouse cells upon heat shock (75), although the absolute level found in the cytoplasm does not decrease. Collier and Schlesinger report transport of hsp90 to the cell nucleus in chicken cells only after a second heat shock (15). Association of hsp89 with tubulin and actin (53, 67) as well as with a number of proteins having regulatory functions, such as tyrosine kinase oncogene products (10, 46, 55, 85), steroid receptors (33), and the eIF-2α kinase (63), has been reported. The newly synthesized and as yet unphosphorylated tyrosine kinase oncogene products are selectively associated with hsp89 and a 50-kilodalton polypeptide in a complex having a half-life of about 15 min (11). Phosphorylation and membrane association of the oncogene products coincide with dissociation from the complex (11). The tyrosine kinase activity is suppressed in the complex with hsp89. The steroid receptors are also inactive in DNA binding while associated with hsp89 (30, 58, 66). These observations have led to the suggestion that hsp89 functions in transport of regulatory proteins to their final cellular locations on the membrane or in the nucleus (3, 67). It has also been suggested that hsp89 sequesters regulatory proteins in an inactive form after synthesis (3, 25) until they reach their destination within the cell or until the appropriate signal for their activity is received. Both the α and β forms of hsp89 are modified by phosphorylation at serine residues in vivo (35, 79), and these modifications may influence the interactions of the HSP with other cellular constituents. In vitro, casein kinase II phosphorylates threonine as well as serine in the eIF-2α kinase-associated hsp89 (63). Both forms of human hsp89 have been shown in vivo to contain phosphorylated serine at two specific sites within the highly charged region located between residues 222 and 290 (44a). The same amino acids are also phosphorylated in vitro by casein kinase II. The serines flank one of two adjacent regions of the polypeptide with high α-helical potential which have been proposed to be the site of interaction of hsp89 with steroid receptors and specifically to interdigitate with the DNA-binding "fingers" (3). Phosphorylation of these sites could have a profound effect on the heat shock protein-receptor interaction. The specific amino acid sequence varies in this region of hsp89 among the different species, but the characteristic clustering of charged amino acids is present in all but the *Escherichia coli* hsp89 homolog p62.5. Phosphorylation of human hsp89 by a novel double-stranded DNA-dependent kinase has also been reported (76). This kinase phosphorylates only hsp89α at one or more threonines in the amino-terminal third of the molecule (C. Anderson, personal communication). The phosphorylation site(s) is distinct both from the sites phosphorylated by casein kinase II described above and from the sites phosphorylated in the eIF-2α kinase-associated hsp89 (63). Identification of phosphorylation sites that are unique to either the α or β form of hsp89 may provide clues toward understanding the possibly different biological roles of the two very similar proteins.

The genes encoding the hsp89 proteins have been shown to be under complex regulation, responding independently to nutritional status of the cells (41, 79), developmental events (2, 4, 52), hormone stimulation (59), and viral infection (70) as well as to the variety of stresses that induce the full complement of stress proteins. The *hsp89α* gene we have isolated has been shown specifically to be induced by heat stress, in response to the adenovirus E1A gene product, and after addition of fresh serum. The constitutive level of this transcript is controlled by growth factors contained in serum, as might be expected for an mRNA encoding an abundant cytoplasmic protein. Thus, the protein accumulates in concert with the general increase in cytoplasmic mass that occurs during proliferation. Although transcription of both *hsp70* and *hsp89α* genes are induced by serum, the steady-state level of *hsp89α* mRNA found in exponentially growing cells is considerably higher. Consequently, hsp89 is synthesized in unstressed cells to a much greater extent than is hsp70. We have shown that this difference can be explained by the longer half-life of *hsp89* mRNA during normal cell growth conditions in which *hsp70* mRNA turns over rapidly (73). Our results do not rule out the possibility of other forms of the *hsp89* gene that contribute to the mRNA pool during normal growth conditions or during stress. Such genes might be regulated differently than the *hsp89α* gene that was studied. However, unless the other genes contribute only minor amounts of transcript, they must be virtually identical to the lambda 86 gene or else completely fail to hybridize with any of the probes that were used. Isolation and analysis of the other members of the *hsp89* gene family will be necessary to definitively determine whether they encode other types of hsp89 proteins distinct from the α and β forms or whether they are inactive pseudogenes.

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