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Structure and Expression of the Human Gene Encoding Major Heat Shock Protein HSP70

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Received 9 October 1984/Accepted 16 November 1984

We have cloned a human gene encoding the 70,000-dalton heat shock protein (HSP70) from a human genomic library, using the Drosophila HSP70 gene as a heterologous hybridization probe. The human recombinant clone hybridized to a 2.6-kilobase polyadenylated mRNA from HeLa cells exposed to 43°C for 2 h. The 2.6-kilobase mRNA was shown to direct the translation in vitro of a 70,000-dalton protein similar in electrophoretic mobility to the HSP70 synthesized in vivo. From the analysis of S1 nuclease-resistant mRNA-DNA hybrids, the HSP70 gene appears to be transcribed as an uninterrupted mRNA of 2.3 kilobases. We show that the cloned HSP70 gene contains the sequences necessary for heat shock-induced expression by two criteria. First, hamster cells transfected with a subclone containing the HSP70 gene and flanking sequences synthesized a HSP70-like protein upon heat shock. Second, human cells transfected with a chimeric gene containing the 5' flanking sequences of the HSP70 gene and the coding sequences of the bacterial chloramphenicol acetyltransferase gene transcribed the chimeric gene upon heat shock. We show that the HSP70 mRNA transcribed in an adenovirus 5 transformed human cell line (293 cells) is identical to the HSP70 mRNA induced by heat shock.

Human tissue culture cells respond to heat shock, and certain other stimuli, by the induced synthesis of a small set of proteins (molecular weights 100,000, 70,000, and 37,000 [58]). The effect of heat shock on the pattern of protein synthesis in human cells is similar to that for other eucaryotic cells (reviewed in reference 55). This highly conserved response—the activation of a small number of genes and the repression of other normally active genes—has been most intensively studied in Drosophila (1). The altered protein synthetic pattern is, in general, a reflection of both the preferential transcription of heat shock genes and the selected translation of their mRNAs.

The major heat shock protein synthesized by eucaryotic cells belongs to a family of 70,000-dalton proteins (HSP70). The conservation of HSP70 among species is revealed by the similar sizes, apparent isoelectric points, and tryptic peptide patterns (64). Indeed, polyclonal antibodies raised against chicken HSP70 cross-react with proteins of similar size from yeast, Drosophila, Xenopus, mice, and humans (32). In Drosophila, the HSP70 multigene family encodes two heat shock proteins, HSP68 and HSP70 (25), and three cognate proteins (27) whose synthesis occurs at normal temperature and which are not heat shock inducible. Drosophila melanogaster has five copies of the HSP70 gene, two at the 87A chromosomal locus and three at the 87C locus (25). Similarly, Saccharomyces cerevisiae contains two copies of the HSP70 gene (28).

The sequence conservation of the HSP70 genes among species has been used to isolate the homologous genes from yeast (28) and chickens (R. Morimoto, C. Hunt, L. Berg, and B. Wu, Proc. Natl. Acad. Sci. U.S.A., in press), using the Drosophila HSP70 gene as a heterologous hybridization probe. We have utilized this homology to isolate a genomic clone containing a human HSP70 gene. Human cDNA clones encoding the 70-kilodalton (kd) heat shock proteins have been reported by Cato et al. (9) and Kao and Nevin (31). In this study, we describe the structural features of the human HSP70 gene: the organization of the genomic clone and the location of the 5' and 3' termini of the heat shock-induced HSP70 transcript. We examine the expression of the human HSP70 gene in hamster cells and of a chimeric HSP70 bacterial chloramphenicol acetyltransferase (CAT) gene in human cells.

MATERIALS AND METHODS

General methods. The human genomic lambda library (39) was generously provided by T. Maniatis. Approximately 5 × 107 recombinant phage were screened (22) for sequences homologous to a subclone (plasmid 232.1; 41) containing 1.1 kilobase (kb) of coding sequence adjacent to the 5' end of the Drosophila HSP70 gene. Hybridizing plaques were purified, and DNA was isolated from plaque particles by equilibration CsCl centrifugation (42).

Genomic DNA was prepared from human placental tissue lysed with sodium dodecyl sulfate (SDS) and digested with proteinase K (8). Subclones of H3-1 were constructed with the vector pBR322 or pAT153 and used to transform Escherichia coli HB101. Plasmid DNAs were isolated by ethidium bromide-cesium chloride equilibrium centrifugation (41). DNAs were radioactively labeled with [α-32P]deoxyribonucleoside triphosphates (Amersham Corp.) by nick translation (52).

Restriction mapping, gel electrophoresis, and gel blotting. Restriction enzyme digestions of DNA were done under conditions recommended by the suppliers (New England BioLabs, Bethesda Research Laboratories, and Promega Biotech). Restriction fragments were separated by agarose (60) or polyacrylamide (43) gel electrophoresis. Southern blot (59) replicas were prepared, and the filters were prehybridized for 8 h at 65°C in a solution containing 6× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.4), 5× Denhardt solution (1× is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), and 100 µg of sheared herring sperm carrier DNA per ml.

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The radiolabeled DNAs were denatured by boiling for 5 min, chilled on ice, and added to the prehybridization solution for an additional 12 to 16 h of incubation. The filters were subsequently washed in 0.2X SSC and 0.1% SDS for 2 h at 65°C, and autoradiograms were obtained after exposure to Kodak XAR film at -70°C.

**Isolation of RNA and Northern blot analysis.** HeLa and 293 cells were grown in suspension to a density of 3 x 10⁴ to 6 x 10⁶ cells per ml in Joklik’s modification of Dulbecco modified Eagle (DME) medium supplemented with 5% fetal calf serum. Cells were either maintained at 37°C or heat shocked at 43°C for 2 h and were collected by centrifugation. Cytoplasmic polyadenylated [poly(A)+] mRNAs were isolated from cell lysates (12) by oligo-deoxythymidylate chromatography (2).

The poly(A)+ mRNA samples were glyoxylated, electrophoretically separated on neutral agarose gels, and transferred onto nitrocellulose (61). The filters were prehybridized for 8 h at 42°C in a solution containing 6X SSC, 0.2% SDS, 50% formamide, 5X Denhardt solution, and 100 μg of carrier DNA per ml. The denatured radiolabeled plasmid DNA was added, and hybridization was continued for 16 h. The filters were subsequently washed in 0.2X SSC and 0.2% SDS for a total of 3 h at 65°C.

**mRNA selection and in vitro translation.** A modification of the hybridization-selection and translation technique (51) was used for mRNA selection and in vitro translation. One microgram of pH2.3 or plasmid vector pAT153 was linearized by digestion with BamHI, denatured in 0.1 M NaOH, and transferred onto nitrocellulose with a Miniport apparatus (Schleicher & Schuell, Inc.). The filters containing bound plasmid DNAs were excised, baked in vacuo for 2 h at 80°C, rinsed twice with boiling water, and prehybridized as previously described. The filters were hybridized with 10 μg of poly(A)+ mRNA from control or heat-shocked HeLa cells in a solution containing 65% formamide, 0.4 M NaCl, 40 mM PIPES[piperazine-N,N'-bis(2-ethanesulfonic acid)] (pH 6.8), and 2 mM EDTA at 50°C for 3 h. A separate filter containing the plasmid pAT153 was also placed in each hybridization reaction to serve as a negative control. The filters were subsequently washed repeatedly with 0.1 M NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, and 0.5% SDS at 65°C. The hybridized transcripts were released with 10 mM methyl mercury hydroxide and precipitated with ethanol. The pellet was repeatedly washed with 70% ethanol, suspended in water, and used for in vitro translation with micrococcal nuclease-treated rabbit reticulocyte lysates (50). The [35]S)methionine-labeled translation products were analyzed by electrophoresis on 10% polyacrylamide gels containing SDS (36) and by fluorography (38).

**Mapping the 5' and 3' termini of the HSP70 transcript.** The boundaries of the transcriptional unit were determined by S1 nuclease (7) and primer extension (37) analyses. DNA templates for mapping the 5' terminus were prepared by restriction enzyme digestion of the appropriate subcloned DNA fragments. The 5' termini were dephosphorylated with 40 U of calf intestinal phosphatase (Boehringer Mannheim Biochemicals) per ml at 37°C for 1 h in 50 mM Tris-hydrochloride (pH 9.0)–1 mM MgCl2–0.1 mM ZnCl2–1 mM spermidine. The dephosphorylation reaction was quenched with an equal volume of 10 mM Tris-hydrochloride (pH 8.0)–0.1 M NaCl–1 mM EDTA–0.5% SDS, heated to 68°C for 15 min, and extracted with phenol-chloroform (1:1). The 5' termini were phosphorylated with [γ-32P]ATP (3,000 Ci/mmol; Amer sham) and T4 polynucleotide kinase (44). Unincorporated nucleotides were removed by Sephadex G-50 chromatography.

The plasmid pH2.8, 5' end-labeled at the BamHI site, was subsequently digested with AvaI, and the 1.3-kb BamHI-AvaI fragment was purified by electroleution from agarose gels. Templates for mapping the 3' terminus were prepared by selective 3' end-labeling, using the Klonef fragment of E. coli DNA polymerase I and the appropriate [α-32P]deoxynucleoside triphosphate (16). The plasmid pH2.3 was 3' end-labeled at the BamHI site, resulting in a 5.6-kb template. The 520-base pair (bp) HindIII-AvaI template was prepared by digesting pH2.3 with BamHI and AvaI and 3' end-labeling. The 2.0-kb AvaI fragment was isolated and digested with HindIII to generate a 520-bp fragment from the insert and a 1.4-kb fragment from the vector. Each template was mixed with 10 μg of poly(A)+ mRNA from control or heat-shocked HeLa cells or with tRNA in a hybridization solution of 80% formamide–0.4 M NaCl–40 mM Pipes (pH 6.8)–2 mM EDTA. The hybridization mixture was incubated at 68°C for 15 min and for 4 h at 54°C. The reaction mix was diluted with 3 volumes of 66 mM sodium acetate (pH 4.5)–4 mM Na2SO4–0.3 M NaCl and incubated with 500 U of S1 nuclease (Boehringer-Mannheim) per ml for 1 h at 37°C. The S1 nuclease-resistant DNAs were denatured and analyzed by alkaline–1.2% agarose (45) or 8.3 M urea–4% polyacrylamide gel electrophoresis and autoradiography.

We used the primer extension method to confirm the location of the 5' terminus. A 60-bp BamHI-PstI fragment, 5' end-labeled at the BamHI site, was purified from plasmid pH2.8 by polyacrylamide gel electrophoresis (44). The radiolabeled primer was mixed with 10 μg of poly(A)+ mRNA from control or heat-shocked cells or with tRNA, denatured, and allowed to hybridize under conditions used in the S1 nuclease assay. The hybridization mixture was diluted with distilled water, and the nucleic acids were precipitated with ethanol. The pellet was suspended in 0.3 M sodium acetate, ethanol precipitated, and repeatedly washed with 70% ethanol. The hybridized radiolabeled primer was elongated by incubation at 37°C for 1 h in 50 mM Tris (pH 8.3)–50 mM KCl–10 mM dithiothreitol–10 mM MgCl2–40 μg of actinomycin D per ml–600 μM of each deoxyribonucleotide triphosphates–6 U of reverse transcriptase. The RNA template was degraded by incubation in 0.1 M NaOH for 10 min at 68°C. After neutralization with 0.3 M sodium acetate (pH 5.2), the DNA was extracted with phenol-chloroform (1:1) and 0.1% SDS and ethanol precipitated. Unincorporated nucleotides were removed by Sephadex G-50 chromatography. The primer extension products were analyzed by 8.3 M urea–4% polyacrylamide gel electrophoresis and autoradiography.

**Cell culture and transfection.** Hamster V79 (a gift from E. H. Y. Chu), HeLa, and 293 (21) cells were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. At 24 h before transfection, V79 and HeLa cells were plated at a density of 10⁶ cells per cm². The cells were exposed to the DNA and calcium phosphate precipitate, prepared as described by Wigler et al. (65), for 4 to 6 h in the case of V79 cells and for 24 h in the case of 293 cells. The cells were washed with warmed phosphate-buffered saline, and fresh medium was added. After 48 h, cultures were divided for the analysis of proteins or RNA. Proteins were labelled in vivo with [35]S)methionine (4) from control and heat-shocked (43°C for 1 h) V79 cells transfected with plasmid pHF6.3 and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Total cellular RNAs were isolated, by a modified guanine thiocyanate procedure (10) followed by hot phenol extraction (17), from control and heat-shocked (43°C for 2 h) 293 cells transfected with pHBCAT. The cellular RNAs were used to protect pHBCAT.
BamHI-HindIII, and HpaII, and the H3-1 homologous sequences were cloned into the gene and HindIII, homologous sequences sites for the gene double restriction determined from digested agarose fragments. Small H3-1 homologous sequences with PstI identified; bacteriophage with HindIII-BamHI digestion products were 5' nuclease-resistant fragments and clones were identified. Of the four restriction enzyme sites for the gene, cloned into Charon 322.1 (plasmid 232.1; [41]).

The regions that hybridized to the Drosophila HSP70 gene, plasmid 232.1 (41), are indicated above the H3-1 map. The Smal fragments are bracketed and lettered in correspondence to size.

5' end-labeled at the EcoRI sites in an S1 nuclease assay. S1 nuclease-resistant fragments were analyzed by urea-polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Isolation of a recombinant human clone containing sequences homologous to the Drosophila HSP70 gene. A recombinant DNA library (10^5 phage) of human genomic DNA cloned into the bacteriophage vector Charon 4A (39) was screened by hybridization with 32P-labeled DNA containing the coding sequences from the 5' end of the Drosophila HSP70 gene (plasmid 232.1; [41]). Four positive recombinant clones were identified; each was subsequently plaque purified and reexamined for cross-homology to the Drosophila HSP70 gene. Of the four recombinant clones that contained sequences homologous to the Drosophila HSP70 gene, two independent isolates (H3-1 and H4-1) were found to have identical sites for the restriction enzymes EcoRI, BamHI, HindIII, and Smal. Clone H3-1 was chosen for further studies. The restriction map of the 17-kb insert (Fig. 1) was determined from analysis of combinations of single and double restriction enzyme digestion products separated by agarose and polyacrylamide gel electrophoresis.

The regions of H3-1 homologous to the Drosophila HSP70 gene were identified by Southern blot analysis. H3-1 DNA was digested with HindIII-BamHI or Smal, separated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with 32P-labeled 232.1 (Fig. 2). Two fragments in each restriction enzyme digestion showed homology to the heterologous probe: the 3.8- and 2.8-kb Smal fragments and the 2.3- and 1.2-kb HindIII-BamHI fragments. These Smal fragments are contiguous, and their map positions overlap with the locations of the 2.3- and 1.2-kb HindIII-BamHI fragments (Fig. 1). However, because these HindIII-BamHI fragments are separated by a 2.8-kb BamHI fragment, we conclude that the two regions in H3-1 homologous to the Drosophila HSP70 gene are discontinuous.

We examined the sequence relationship between the 2.3- and 1.2-kb HindIII-BamHI fragments by subcloning each fragment into the plasmid vector pAT153. These subclones were 32P labeled and hybridized to the same restriction digests of H3-1 (Fig. 2). The subclone pH2.3 hybridized strongly, as expected, to the 2.3-kb HindIII-BamHI fragment in H3-1 and weakly to the 1.2-kb HindIII-BamHI fragment. These results suggest that sequences in the 2.3- and 1.2-kb HindIII-BamHI fragments are related but not identical. This conclusion is supported by the reciprocal experiment. The subclone pH1.2 hybridized strongly, as expected, to the 1.2-kb HindIII-BamHI fragment in H3-1 and weakly to the 2.3-kb HindIII-BamHI fragment. In addition, pH1.2 hybridized to many other DNA fragments in H3-1 (Fig. 2), suggesting that the 1.2-kb HindIII-BamHI fragment contains a repetitive sequence that is dispersed throughout the 17-kb insert in H3-1. Because the 2.8-kb
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BamHI fragment hybridized by the pH1.2 probe and not with the pH2.3 probe, the homology between the 2.8-kb BamHI and the 1.2-kb BamHI-HindIII fragments may reside in shared repetitive sequences.

Genomic organization of the human DNA sequences cloned in H3-1. The genomic organization of sequences in the entire H3-1 clone and subcloned regions was determined by Southern blot analysis of human genomic DNA digested with BamHI-HindIII and hybridized with 32P-labeled H3-1 or H3-1 subclones (Fig. 1). H3-1 as well as the fragments subcloned in pH7.0, pH11.0, pH2.8, and pH1.2 each hybridized to many DNA fragments, resulting in a smear on the autoradiogram (Fig. 3A). This pattern of hybridization is expected only if each of the hybridization probes contains repetitive sequences. The subclone pH2.3 hybridized to the expected 2.3-kb fragment and also to a 6.0-kb fragment of equivalent autoradiographic intensity. (This 6.0-kb fragment is smaller than the 6.3-kb HindIII fragment in H3-1 and in genomic digests; therefore it does not appear to be the result of partial digestion.) Other fragments, 4.8, 4.0, and 3.0 kb in size, hybridized weakly to pH2.3. These hybrids were stable even after stringent washing with 0.1× SSC at 65°C and 3 mM Tris base at room temperature. The thermostability of these hybrids between pH2.3 and the genomic fragments suggests that sequences in pH2.3 are conserved and repeated in the genome.

The copy number of the 2.3-kb BamHI-HindIII fragment in human genomic DNA was determined by comparing its autoradiographic intensity with that of marker copy equivalents by Southern blot hybridization. Assuming the diploid genome size to be ca. 109 bp, a single copy of a 2.3 × 102-bp fragment per haploid genome would be the equivalent of 92 pg in 20 μg of genomic DNA. The hybridization intensity of the 2.3-kb fragment in genomic DNA is similar to that of the single-copy equivalent of the 2.3-kb fragment in pH6.3 (Fig. 3B). Therefore, there is a single copy of the 2.3-kb fragment per haploid genome. The 6.0-kb fragment appears to be present at one or two copies per haploid genome.

Localization of dispersed repetitive sequences in H3-1. The approximate locations of the repetitive sequences scattered throughout H3-1 was determined by hybridization of 32P-labeled genomic DNA to a Southern blot containing Smal fragments of H3-1, an approach that was also used by Shen and Maniatis (57) to detect repetitive sequences in the rabbit β-like globin gene cluster. In this assay, the autoradiographic intensity of hybridizing fragments should reflect the relative copy number of those sequences in the genome. For example, from the relative intensities of hybridization shown in the autoradiogram (Fig. 4A), Smal fragments b, c, e, and f are at least 5- to 10-fold more repetitive in the genome than is the Smal fragment d. The relative intensities are consistent with the results of the Southern blot analyses (Fig. 3). For example, Smal fragment d corresponds to the map position of the 2.3-kb BamHI-HindIII fragment.

Replica filters containing Smal fragments of H3-1 were hybridized with subclones pHH1.0, pHH1.2, and pHH2.8 to determine whether the repetitive sequences within each subclone were related. Each subclone hybridized with the expected Smal fragment(s) as predicted by the restriction map of H3-1 (Fig. 4B). In addition, each subclone also hybridized to other Smal fragments as indicated by arrows (Fig. 4A). The nonpredicted fragments are shared among the subclones, suggesting that the dispersed repetitive sequences in each subcloned fragment are related. The differ-

FIG. 2. H3-1 contains two regions that cross-hybridize with the Drosophila HSP70 gene. H3-1 DNA was digested by BamHI-HindIII (B + H) or by Smal (S). Fragments were separated on a 1.2% agarose gel, transferred onto nitrocellulose, and hybridized with 32P-labeled H3-1, Drosophila HSP70 gene 232.1, pH2.3 (Fig. 1) or pH1.2 (Fig. 1). The Drosophila gene hybridized to 2.3- and 1.2-kb HindIII-BamHI fragments and 3.8- and 2.8-kb Smal fragments. The sizes of fragments are given in kb.

FIG. 3. Genomic organization of DNA sequences in H3-1. (A) Human genomic DNA was digested with BamHI-HindIII, electrophoretically separated on a 1.2% agarose gel, transferred onto nitrocellulose, and hybridized with 32P-labeled H3-1, pH7.0, pH11.0, pH2.8, or pH1.2 as indicated. (B) Dosage analysis of the 2.3-kb BamHI-HindIII fragment in human genomic DNA (H) by comparison with copy equivalents of pH6.3 digested with BamHI-HindIII at one copy per haploid genome (1c) and two copies per haploid genome (2c). The filter was probed with 32P-labeled pH2.3. One and two copies per haploid genome of the 2.3-kb fragment corresponds to 400 and 800 pg, respectively, of pH6.3.
sequences in the intensities of the fragments among different probes may reflect either the degree of homology or the extent of reiteration of the repetitive sequence.

Identification of a heat shock-induced mRNA homologous to the 2.3 kb HindIII-BamHI fragment and adjacent sequences. Transcripts homologous to the 2.3-kb HindIII-BamHI fragment and adjacent sequences were identified by hybridization of each 32P-labeled subclone to Northern blots. Cytoplasmic poly(A)+ mRNAs were isolated from control (37°C) and heat-shocked (43°C for 2 h) HeLa cells, glyoxalated, electrophoretically separated on neutral agarose gels, and transferred onto nitrocellulose (62). Replica filters, each containing mRNAs from control and heat-shocked cells, were hybridized with 32P-labeled DNAs corresponding to the two regions homologous to the Drosophila HSP70 gene (pH2.3 and pH1.2) and the intervening fragment (pH2.8). pH2.3 is homologous to a 2.6-kb mRNA induced in heat-shocked cells (Fig. 5). Consistent with the level of homology between the 1.2- and 2.3-kb BamHI-HindIII fragments, pH1.2 hybridized weakly to a 2.6-kb heat shock-induced transcript. Sequences in pH1.2 also hybridized to multiple high-molecular-weight mRNAs from control and heat-shocked cells, resulting in a smear on the autoradiogram. We presume that this result was due to hybridization with the repetitive element in pH1.2. As predicted by the shared repetitive sequences in the 2.8-kb BamHI and the 1.2 BamHI-HindIII fragments, pH2.8 hybridized to many high-molecular-weight mRNAs from control and heat-shocked cells. Sequences in pH2.8 also hybridized to a 2.6-kb heat shock-induced transcript. The 2.8-kb BamHI and the 2.3-kb BamHI-HindIII fragments are not homologous to each other, yet both hybridize to a 2.6-kb heat-inducible mRNA. Therefore, the data suggest that the transcription unit for the 2.6-kb mRNA spans at least these two adjacent fragments.

The 2.6-kb heat shock-induced mRNA codes for HSP70. The protein translated from the 2.6-kb poly(A)+ mRNA.

FIG. 4. Sequence relatedness of the repetitive elements surrounding the HSP70 gene in H3-1. (A) Southern blot analysis of H3-1 digested with SmaI and hybridized with 32P-labeled human placental DNA, pH11, pH2.8, or pH1.2 as indicated. Arrows point to fragments not expected, based on their map positions (shown in B), to have homology with the given probe. (B) The locations of SmaI fragments of H3-1, subclones used for hybridization, and the positions of the repetitive elements are shown.
induced in heat-shocked HeLa cells was identified by hybridization-selection and in vitro translation (51). The 2.3-kb HindIII-BamHI fragment was used to select the 2.6-kb heat-inducible mRNA for in vitro translation. We have previously shown that pH2.3 only hybridized to a 2.6-kb, heat shock-induced mRNA. The DNA of subclone pH2.3 was linearized, denatured, transferred onto nitrocellulose, and hybridized with poly(A)$^+$ mRNA from control cells or heat-shocked HeLa cells. A separate filter containing the plasmid vector pAT153 was also placed in each hybridization reaction to serve as a negative control. The filters were subsequently washed, and the hybridized transcripts were released and translated in rabbit reticulocyte lysates. The $^{[35S]}$methionine-labeled translational products were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 6). The 2.6-kb mRNA selected by hybridization with pH2.3 directed the translation in vitro of a 70,000-dalton protein whose electrophoretic mobility by one-dimensional SDS-polyacrylamide gel electrophoresis was similar to that of the in vivo-synthesized HSP70 in heat-shocked HeLa cells. HSP70 was not translated from either control or heat shock mRNA samples selected by the plasmid vector pAT153. Consistent with the Northern analysis, a low level of HSP70 synthesis from control mRNA samples selected by pH2.3 is detected only after prolonged exposure to X-ray film. The HSP70 message is enriched in heat-shocked cells.

Mapping the 5' and 3' boundaries of the HSP70 transcription unit. We inferred from the results of the Northern blot analysis that the HSP70 transcription unit spanned at least the two adjacent 2.8-kb BamHI and 2.3-kb BamHI-HindIII fragments. The two possible directions of transcription were examined by preparing the 2.8-kb BamHI fragment, 3' or 5' end-labeled at the BamHI sites. Each radiolabeled template was denatured and hybridized to poly(A)$^+$ mRNA from control or heat-shocked HeLa cells. The RNA-DNA hybrids were subsequently digested with S1 nuclease, and the resistant fragments were analyzed by alkaline-agarose and urea-polyacrylamide gel electrophoresis (data not shown). Because only the 5' end-labeled template was protected from S1 nuclease digestion by poly(A)$^+$ mRNA from heat-shocked HeLa cells, the direction of transcription must be from the 2.8-kb BamHI fragment towards the 2.3-kb BamHI-HindIII fragment.

We located the 5' terminus of the HSP70 transcription unit within the 2.8-kb BamHI fragment by using two methods, S1 nuclease digestion (7) and primer extension (37). The map positions of the templates or primers used in the following experiments are shown in the schematic diagram in Fig. 7. A 1.3-kb BamHI-AvaI fragment isolated from pH2.8 was 5' end-labeled at the BamHI site. The template was denatured and hybridized to poly(A)$^+$ mRNA from control or heat-shocked HeLa cells, and the RNA-DNA hybrids were...
AvaI template product. The extension urea-polyacrylamide gel HeLa cells heat-shocked resistant fragments subsequently digested with S1 nuclease. The S1 nuclease-resistant fragments were analyzed by alkaline-agarose and urea-polyacrylamide gel electrophoresis. The 1.3-kb BamHI-AvaI template was protected by poly(A)+ mRNA from heat-shocked HeLa cells to yield two S1 nuclease-resistant fragments of 180 and 150 bp (Fig. 8A). Both S1-resistant fragments are present at reduced levels when poly(A)+ mRNA from control HeLa cells is used to protect the template, and they are not detected in the absence of mRNA.

![Diagram of S1 nuclease assay](image)

**FIG. 7.** Schematic diagram of the locations of the templates and the respective S1 nuclease-resistant fragments and the primer and its extension product. The broken line represents vector pAT153. Parts A to D correspond to panels A to D in Fig. 8.

![Mapping diagram](image)

**FIG. 8.** Mapping the 5' and 3' boundaries of the HSP70 transcription unit by S1 nuclease (A,C,D) and by primer extension (B) methods. The templates (t) for S1 nuclease assays or primer (p) for primer extension assay were (A) 1.3-kb BamHI-AvaI fragment isolated from pH2.8, 5' end-labeled at the BamHI site; the shorter 1.1-kb fragment is the BamHI-AvaI fragment of the vector pAT153; (B) 60-bp BamHI-PstI fragment isolated from pH2.8, 5' end-labeled at the BamHI site; (C) 5.6-kb BamHI fragment prepared from pH2.3, 3' end-labeled at the BamHI sites; and (D) 520-bp HindIII-AvaI fragment isolated from pH2.3, 3' end-labeled at the AvaI site. The larger fragments are the 2.0 AvaI fragment uncut by HindIII and the 1.4-kb AvaI-HindIII fragment of the vector pAT. The assays were done with poly(A)+ mRNA from control (c) or heat-shocked (hs) HeLa cells or in the absence of mRNA (-). Arrows indicate the S1-resistant fragments or primer extension product. Molecular length markers (m) for A and B are 3' end-labeled HindII fragments of pBR322 and for C are 3' end-labeled HindIII fragments of lambda DNA. The locations of the templates and the respective S1 nuclease-resistant fragments and the primer and its extension product are shown in the schematic diagram in Fig. 7.
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FIG. 9. Expression of human HSP70 in transfected hamster cells. (A) Human HSP70 is distinct from hamster HSP72. Shown is an autoradiogram of [35S]methionine-labeled proteins from control (c) and heat-shocked (hs) HeLa and V79 cells analyzed by SDS-acrylamide gel electrophoresis. "mix" indicates a mixture of proteins from control HeLa and V79 and heat-shocked HeLa and V79 cells. (B) A protein similar in size to HSP70 synthesized in heat-shocked HeLa cells is synthesized in heat-shocked pH6.3-transfected V79 (+pH6.3) cells but not in mock-transfected cells (mock). Two independent heat-shocked and pH6.3-transfected samples are shown.

To confirm the location of the 5' terminus, we used a complementary approach: the method of reverse transcriptase-catalyzed primer extension. If there are indeed two 5' termini, as suggested by the protection of the 180- and 150-bp fragments from S1 nuclease digestion, then we expect to detect two discrete cDNAs. A 60-bp BamHI-PstI primer isolated from pH2.8 was 5' end-labeled at the BamHI site, denatured, and hybridized to poly(A)+ mRNA from control of heat-shocked HeLa cells. The primer-RNA hybrid was elongated in the presence of deoxynucleoside triphosphates and reverse transcriptase. The RNA was subsequently degraded by incubation in alkali, and the cDNA products were analyzed by urea-polyacrylamide gel electrophoresis. The 60-bp primer was elongated by reverse transcriptase to a single 150-bp fragment corresponding to the location of the 5' terminus from the radiolabeled BamHI site (Fig. 8B). An elevated level of the 150-bp cDNA was reverse transcribed from poly(A)+ mRNA from heat-shocked HeLa cells relative to that transcribed from poly(A)+ mRNA from control HeLa cells. We have not investigated the source of the 180-bp fragment detected by the S1 nuclease assay. We conclude that the 5' terminus of the HSP70 transcript lies 150 bp upstream of the BamHI site at the junction of the 2.8-kb BamHI and the 2.3-kb BamHI-HindIII fragments.

The 3' terminus of the HSP70 transcription unit was located by using pH2.3, 3' end-labeled at the BamHI site, as the template for hybridization with poly(A)+ mRNA from control or heat-shocked HeLa cells. The RNA-DNA hybrids were digested with S1 nuclease, and the S1 nuclease-resistant fragments were analyzed by acrylamide gel electrophoresis. This template was protected by poly(A)+ mRNA from heat-shocked HeLa cells to yield a 350-bp, S1 nuclease-resistant fragment (Fig. 8D). A reduced level of the 350-bp, S1 nuclease-resistant fragment was protected by poly(A)+ mRNA from control HeLa cells. Thus, both templates positioned the 3' terminus at approximately the same location (Fig. 7).

We conclude from the positions of the 5' and 3' termini that the 2.6-kb mRNA coding for HSP70 may be transcribed as a 3.3-kb uninterrupted transcript. (Nucleotide sequence analysis has confirmed the locations of the 5' and 3' termini; C. Hunt and R. Morimoto, manuscript in preparation.) The difference of 300 bp between the size of our deduced primary transcript and the size of the mature poly(A)+ mRNA is likely to be the 3'-terminal poly(A)+ tail.

Expression of HSP70 in transfected hamster cells. To prove that the cloned HSP70 gene contained the regulatory sequences for heat shock-induced expression of HSP70, we introduced the subclone pH6.3, containing the HSP70 gene and flanking sequences, into hamster cells. Hamster cells were chosen as the recipient cells because the human HSP70 is distinct in size from hamster HSP72 by one-dimensional SDS-polyacrylamide gel electrophoresis (Fig. 9A) and by two-dimensional gel electrophoresis (data not shown). Therefore, the differences in the electrophoretic mobilities between the hamster and human heat shock proteins can be used to examine the expression of the human HSP70 gene in hamster cells. Hamster V79 cells were exposed to calcium phosphate precipitates containing pH6.3 (V79 + pH6.3) or the plasmid vector pAT153 (mock). At 48 h posttransfection, the cultures were either maintained at 37°C or heat shocked for 1 h at 43°C. The synthesis of HSP70 was measured by pulse-labeling control and heat-shocked transfected hamster cells with [35S]methionine and analyzing the labeled proteins by SDS-polyacrylamide gel electrophoresis and fluorography. A protein with electrophoretic mobility similar to that of human HSP70 was synthesized only in hamster cells transfected with pH6.3 and subsequently heat shocked (Fig. 9B). We conclude that the sequences contained in subclone pH6.3 provide sufficient information for the heat shock-regulated synthesis of a 70,000-dalton protein electrophoretically similar to HSP70. Because the 3' boundaries of pH6.3 and pH2.3 are the same, we can also conclude that most if not all of HSP70 is encoded by sequences within the boundaries of pH2.3.

Expression of HSP-CAT in transfected 293 cells. We reasoned that the sequences necessary for heat shock-induced transcription of the HSP70 gene should lie within pH2.8. First, the 5' terminus of the HSP70 transcript is located within pH2.8, 150 bp from one boundary; therefore, pH2.8 contains 2.6 kb of 5' flanking sequences. Second, the subclone pH6.3 introduced into hamster cells resulted in the heat shock-induced expression of HSP70; therefore, pH6.3 contains the sequences for heat shock-induced expression. Because the 5' flanking sequences in pH6.3 and pH2.8 are the same, we reasoned that sequences in pH2.8 might confer heat shock-regulated transcription of a test gene. A chimeric gene containing the 5' flanking sequences of the HSP70 gene and the coding sequences of the bacterial CAT gene was constructed by inserting the 2.8-kb Bam HI fragment of pH2.8 into the BamHI site of puC-CAT (a gift from R. Kingston), a derivative of the promoterless pSVoCAT (20). The resulting chimeric gene pHB-CAT was introduced into 293 cells by calcium phosphate transfection. At 48 h post-
transfection, total RNA was isolated from transfected cells maintained at 37°C or heat shocked for 2 h at 43°C. The RNAs were hybridized with pHBCAT and 5' end-labeled at the EcoRI sites, and the RNA-DNA hybrids were subsequently digested with S1 nuclease. The S1 nuclease-resistant fragments were analyzed by urea-polyacrylamide gel electrophoresis. The chimeric HSP-CAT transcript initiated at the 5' terminus of the human HSP70 gene should protect a 400-bp S1 nuclease-resistant fragment. An elevated level of a 400-bp S1 nuclease-resistant fragment was protected by RNA isolated from transfected and heat-shocked 293 cells relative to that protected by RNA isolated from transfected 293 cells (Fig. 10). We conclude that the sequences necessary for heat shock-induced transcriptional regulation are upstream of the 5' terminus of the HSP70 gene and are present in pH2.8.

Expression of HSP70 in HeLa and 293 cells. In the adenovirus type 5-transformed human embryonic kidney cell line 293 (21), human HSP70 is constitutively synthesized (48) and the level of synthesis can be elevated by heat shock (Fig. 11). Is the HSP70 mRNA transcribed in non-heat-shocked (control) 293 cells identical to the HSP70 mRNA transcribed in heat-shocked HeLa cells? By Northern hybridization with 32P-labeled pH2.3, we detected a 2.6-kb mRNA in control 293 cells similar in size to the 2.6-kb, heat shock-induced transcript in HeLa and 293 cells (data not shown). Using pH2.3 3' end-labeled at the BamHI site as the template, we found that the S1 nuclease-resistant fragment protected by cytoplasmic mRNA from control 293 cells was similar in size to that protected by cytoplasmic mRNA from heat-shocked HeLa and 293 cells (Fig. 12). The location of the 5' terminus of the HSP70 transcript in control 293 cells was determined by reverse transcription primed with the 60-bp BamHI-PstI fragment isolated from pH2.8 and 5' end-labeled at the BamHI site. The same primer was previously used to locate the 5' terminus of the HSP70 transcript from heat-shocked HeLa cells. The resulting cDNA copied from the HSP70 transcript from control 293 cells is 150 bp (data not shown), similar in size to that copied from the HSP70 transcript from heat-shocked HeLa cells (Fig. 8B). S1 nuclease digestion of the HSP70 transcript from control 293 cells hybridized with the 1.3-kb BamHI-AvaI fragment, 5' end labeled at the BamHI site, resulted in 150- and 180-bp S1 nuclease-resistant fragments (data not shown). Again, these were identical to the fragments protected by the HSP70 transcript from

FIG. 10. Expression of a chimaeric HSP-CAT gene in 293 cells. Shown is an autoradiogram of S1 nuclease-resistant fragments analyzed by urea-polyacrylamide gel electrophoresis. Total RNAs were prepared from control and heat-shocked 293 cells 48 h post-transfection with pHBCAT. The template pHBCAT, 5' end-labeled at the EcoRI sites, is protected by RNA from heat-shocked and transfected 293 cells (hs) to yield a 400-bp S1 nuclease-resistant fragment. A reduced level of this S1 nuclease-resistant fragment is also protected by RNA from control and transfected 293 cells (c). This fragment is not protected by tRNA (—). Molecular length markers (m) are 3' end-labeled pBR322 fragments from a partial HindIII digestion.

FIG. 11. Synthesis of HSP70 is constitutive in 293 cells and increases upon heat shock. Shown is an autoradiogram of [35S]methionine-labeled proteins from control (c) and heat-shocked (hs) HeLa and 293 cells analyzed by SDS-polyacrylamide gel electrophoresis.

FIG. 12. HSP70 mRNA in HeLa and 293 cells. Cytoplasmic mRNA was prepared from 293 and HeLa cells exposed to 43°C for the indicated times and used to protect a 5.6-kb template prepared from pH2.3, 3'end-labeled at the BamHI sites (t) in an S1 nuclease assay. Molecular length markers (m) were provided by the HindIII fragments of lambda phage DNA.
heat-shocked HeLa cells (Fig. 8A). Therefore, we conclude that the HSP70 transcript present in control 293 cells has the same 5' and 3' termini as the HSP70 transcript present in heat-shocked HeLa cells.

**DISCUSSION**

The conservation of the HSP70 genes in eucaryotes (Morimoto et al., in press) has proven effective in the isolation of a human HSP70 gene, in which the *Drosophila* HSP70 gene is used as a heterologous hybridization probe. We have shown that transfection of the cloned human HSP70 gene into hamster cells resulted in the heat shock-induced synthesis of a HSP70-like protein. We have also shown that the regulatory sequences for heat shock-induced expression lie upstream of the HSP70 gene by constructing a chimeric gene containing 2.6 kb upstream and 150 bp of the HSP70 gene fused with the bacterial CAT gene. Transfection of the HSP70-CAT gene into 293 cells resulted in the heat shock-induced transcription of the chimeric gene.

Quite unexpectedly, the human HSP70 gene shares several features with the *Drosophila* HSP68 and HSP70 genes. Our data suggest that the human HSP70 gene belongs to a multigene family, reminiscent of the 70-kd multigene families in *Drosophila* (25, 27, 41) and yeast (28). The coding region of the HSP70 gene is conserved and duplicated elsewhere in the human genome, perhaps corresponding to a nonallelic HSP70 gene. From the analysis of the 5' and 3' termini of the HSP70 mRNA, it appears that the gene is transcribed as an uninterrupted transcript of 2.3 kb. This is further substantiated by DNA sequence analysis of the HSP70 gene (Hunt and Morimoto, in preparation). The colinearity of the human HSP70 gene, though unusual for most eucaryotic genes, except for the histone (reviewed in reference 24) and interferon-α (47) genes, is also seen for *Drosophila* HSP68 and HSP70 genes (25). This structural characteristic may be restricted to the heat-inducible members of the 70-kd multigene family, as at least one member of the *Drosophila* 70-kd cognate genes is interrupted by an intervening sequence (13). The lack of intervening sequences may be a significant rather than a coincidental characteristic for genes whose transcripts accumulate rapidly in the cytoplasm. Certainly the rates of transcription and accumulation of the *Drosophila* HSP70 mRNAs are remarkably rapid (63). Most eucaryotic genes contain intervening sequences that require proper processing in order for the transcript to be transported into the cytoplasm (23). Therefore, modulation of the processing events could regulate transport and cytoplasmic accumulation of mRNAs. For example, in adenovirus-infected human tissue culture cells, cellular RNAs are transcribed at normal rates but are not transported into the cytoplasm (3, 6). If this block is in the processing of the primary cellular RNAs, we would predict that the human HSP70 transcript along with histone and interferon-α transcripts should be able to circumvent this block.

Repetitive sequences flanking the human HSP70 gene were detected by hybridization of radiolabeled genomic DNA to a Southern blot containing restriction digests of H3-1. Reciprocal hybridizations with subcloned fragments revealed that these repetitive sequences are related. Thus, the genetic environment surrounding the human HSP70 gene is similar to that of the *D. melanogaster* HSP70 genes at the 87C chromosomal locus which is flanked by repetitive sequences. Transcription of one such repetitive sequence at 87C is heat inducible (40). The repetitive sequences surrounding the human HSP70 gene hybridize to poly(A) + mRNA from control and heat-shocked HeLa cells. We do not know whether the repetitive sequences present in H3-1 are transcribed at these sites. The dispersal of related repetitive sequences throughout a gene region has been reported for rat casein (66), mouse serum albumin and α-fetoprotein (35), *Xenopus* vitellogenin (33), and rabbit β-like globin (56) genes. Members of the human *alu* family (26, 30, 54) of dispersed repetitive sequences have been found in the cluster of human β-globin genes (18) and distal to the human insulin gene (5). The repetitive sequences surrounding the human HSP70 gene are not present in the hamster genome and are distinct from the human *alu* family, as no homology was detectable by hybridization with the *alu* member cloned in Blur 8 (15; other data not shown). Although functions for dispersed repetitive sequences remain elusive, it has been speculated that these sequences serve as hot spots for recombination (19), exon shuffling, and gene rearrangement (14). The mobility of such sequences is hypothesized to occur via reverse transcription of an RNA intermediate (29). The rearrangements may ultimately result in the species-specific organization of repetitive sequence families which in turn could affect the modulation of gene expression (57).

We have consistently detected low levels of the HSP70 mRNA in non-heat-shocked HeLa cells. This result does not appear to be peculiar to HeLa cells, as similar observations have been reported for other human cell lines (49). We do not know whether the low level of HSP70 expression in non-heat-shocked cells is unique to established cell lines or whether the HSP70 gene is expressed in a subpopulation of the cells, perhaps in a cell cycle-dependent manner.

An elevated level of HSP70 is synthesized in human tissue culture cells after infection with adenovirus 5. This induction is dependent on the adenoviral early gene E1A (48). In adenovirus-transformed human embryonic kidney cells, 293 cells (21), an elevated level of HSP70 synthesis occurs in the absence of heat shock. Presumably the expression of HSP70 in 293 cells is also regulated by E1A. Is the E1A-induced HSP70 mRNA transcribed from the same gene as the heat shock-induced HSP70 mRNA? We have shown that the 5' and 3' termini of HSP70 mRNA isolated from non-heat-shocked 293 cells are similar to those of HSP70 mRNA isolated from heat-shocked HeLa cells. Therefore, it is reasonable to suggest that the cloned HSP70 gene is expressed in non-heat-shocked 293 cells. Alternatively, a duplicated nonallelic HSP70 gene is expressed in non-heat-shocked 293 cells. We favor the former possibility because the chimeric HSP-CAT gene in pHBCAT introduced into 293 cells is transcribed in the absence of heat shock induction. We suggest that the 5' flanking sequences of the cloned HSP70 gene contain sequences that respond to both E1A and heat shock induction.

Recently, Kingston et al. (34) have observed that both E1A and c-myc stimulate the expression of a chimeric hamster dihydrofolate reductase gene containing the 5' sequences of a *Drosophila* HSP70 gene. The stimulation by c-myc occurs only if this 5' region contains sequences between −780 and −200 bp (33) in addition to the *Drosophila* heat shock promoter (51). This upstream region contains repetitive sequences such as a6 (33, 34). If the mechanisms of E1A and c-myc stimulation of HSP70 genes are identical, then the heat shock promoter is not sufficient for transcriptional stimulation by E1A or c-myc. These results are particularly interesting because the human HSP70 gene is also surrounded by repetitive sequences. Treisman et al. (62) have proposed that the differential effect of E1A on the endogenous β-globin gene versus the transfected β-globin gene is due
to chromatin structure. An appealing speculation is that EIA acts as an “uncoiler” of chromatin, the specificity of which may be provided by the repetitive sequences. The uncoiling allows transcription of otherwise quiescent genes. McDougall et al. (46) have proposed that the adenovirus 12-induced gap at human chromosome 17 represents an uncoiled region that is transcribed throughout mitosis.

ACKNOWLEDGMENTS

These studies were supported by grants from the Camille and Henry Dreyfus Foundation, the American Cancer Society, and the Leukemia Foundation. B.W. is a fellow of the Leukemia Society of America, and C.H. is a Public Health Service postdoctoral fellow of the National Institutes of Health.

We thank Carl Wu for stellar discussions, Sheryl Niemiec-Ziemann for advice on message selection, and Daniel Linzer for comments on the manuscript. Sunandita Banerji and Hyeong Reh Choi gathered most of the restriction map data, and Nicholas Theodorakis provided the nuclease-treated lysates.

LITERATURE CITED


