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70-Kilodalton Heat Shock Polypeptides from Rainbow Trout: Characterization of cDNA Sequences

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RTG-2 cells, a line of fibroblasts from rainbow trout (*Salmo gairdnerii*), are induced to synthesize a distinct set of heat-shock polypeptides after exposure to elevated temperature or to low concentrations of sodium arsenite. We isolated and characterized two cDNA sequences, THS70.7 and THS70.14, encoding partial information for two distinct species of 70-kilodalton heat shock polypeptide (hsp70) from these cells. These sequences are identical at 73.3% of the nucleotide positions in their regions of overlap, and their degree of sequence conservation at the polypeptide level is 88.1%. The two derived trout hsp70 polypeptide sequences show extensive homology with derived amino acid sequences for hsp70 polypeptides from *Drosophila melanogaster* and *Saccharomyces cerevisiae*. Northern blot analysis of RNA from arsenite-induced RTG-2 cells, with the trout hsp70 cDNAs as probes, revealed the presence of three hsp70 mRNA species. Southern blot analysis of trout testis DNA cleaved with various restriction endonucleases revealed a small number of bands hybridizing to the hsp70 cDNAs, suggesting the existence of a small family of hsp70 genes in this species. Finally, trout hsp70 cDNA sequences cross-hybridized with restriction fragments in genomic DNA from HeLa cells, bovine liver, *Caenorhabditis elegans*, and *D. melanogaster*.

The heat shock response provides an excellent system for the study of the processes which accompany rapid gene induction in eucaryotic cells. When organisms are subjected to a heat shock, transcription of most genes is suppressed, and the expression of a novel set of proteins is enhanced (for reviews, see references 2, 34, and 39). The induced proteins are termed heat shock polypeptides (hsps). A variety of chemical compounds also elicit the same response (2, 15, 17, 20, 22), suggesting that the heat shock response is probably a reaction to metabolic stress rather than to temperature per se.

Until recently, most data on the heat shock phenomenon were derived from studies with *Drosophila melanogaster*. All organisms studied thus far, however, respond in a similar fashion (34). The highly conserved nature of this response across a wide range of species implies a fundamental role for the hsps in the stressed organism.

The major hsps of most organisms fall into three classes: the small hsps (15 to 30 kilodaltons), the hsp70-like (60 to 70 kilodaltons) polypeptides, and the hsp83-like (80 to 90 kilodaltons) polypeptides. These three classes of hsps are strongly conserved. Antibodies against the hsp70 and hsp89 from chicken embryo fibroblasts cross-react with their counterparts from a wide variety of organisms (16). Similarly, cross-hybridization of hsp70 genes from different organisms has been observed (10, 14, 24, 29, 36). The small hsps from both *D. melanogaster* and *Caenorhabditis elegans* share extensive amino acid sequence homology with the mammalian α-crystallins (12, 33).

The five hsp70 genes from *D. melanogaster* have been cloned and sequenced (11, 13, 30, 42). Very little sequence information about the hsp70 genes from other organisms has been reported. An exception is the report describing a multigene complex of hsp70 genes in *Saccharomyces cerevisiae* (14). The hsp70 sequences from this yeast show extensive homology to the *Drosophila* hsp70 gene.

We have previously reported the induction of hsps by heat shock and sodium arsenite in cultured cells of rainbow trout (*Salmo gairdnerii*) (18). The major stress-inducible protein in trout cells is hsp70. The purpose of the present study was to compare hsp70 gene sequences of trout with those of *D. melanogaster* and *S. cerevisiae*. We report here the isolation and sequencing of two different cDNAs complementary to mRNAs coding for the hsp70 of rainbow trout.

MATERIALS AND METHODS

Cell line and culture conditions. The fibroblast-like line of RTG-2 cells was originally derived from mixed gonadal tissue of male and female rainbow trout (46). Cells were grown in flasks incubated at 22°C. The culture medium consisted of Eagle minimum essential medium supplemented with nonessential amino acids, Earle basic salts, 100 U of penicillin-streptomycin per ml, and 10% fetal bovine serum (all from GIBCO Ltd.). For large-scale growth of cells, roller bottles with a surface area of 300 cm^2 were used. Cells were induced by the addition of sodium arsenite to the culture medium to a final concentration of 50 μM, usually for 24 h (18).

Isolation of RNA. RTG-2 fibroblasts, grown close to confluence, were induced with 50 μM sodium arsenite for 24 h. The cells were subsequently harvested, and the RNA was isolated essentially as described by Chirgwin et al. (6), with a few modifications (18). Briefly, the cell pellets were homogenized by hand on ice with a guanidine · HCl buffer (6 M guanidine · HCl, 20 mM sodium acetate, 0.1 M β-mercaptoethanol, pH 5.0). The homogenates were then carried through one cycle of freeze-thawing, followed by centrifugation at 12,000 × *g* (10 min, 4°C). To the supernatant was added 0.5 volume of 95% ethanol (−20°C). RNA was allowed to precipitate at −20°C for a few hours and was subsequently pelleted by centrifugation at 12,000 × *g* (15 min, 0°C). The pellet was dissolved in 7.5 M guanidine · HCl–25 mM sodium citrate–50 mM β-mercaptoethanol (pH 7.0), and the RNA was precipitated by the addition of 0.025 volume of 1 M acetic acid and 0.5 volume of 95% ethanol (−20°C). This cycle of precipitation was carried out two or three times. The final RNA pellet was washed once with 95% ethanol (−20°C) and then dried under a stream of

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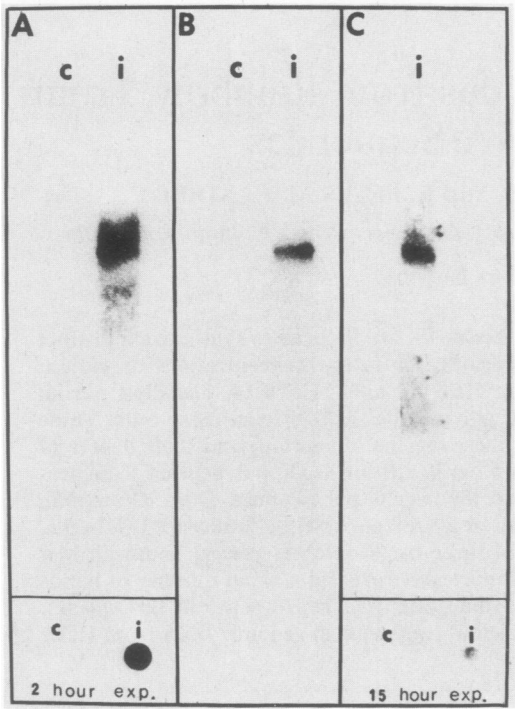

FIG. 1. Northern blot analysis of trout RTG-2 RNA from control (c) and sodium arsenite-induced (i) cells. Either 2 (A and B) or 0.2 μg (C) of poly(A)$^+$ RNA was glyoxalated, separated by electrophoresis on a 1.4% (A and B) or 1.2% (C) agarose gel, and transferred to nitrocellulose filters. The insets to A and C are dot blots of total RNA, 5 μg on each spot. Hybridization was to ^{32}P-labeled THS70.7 (A), THS70.14 (B), or a 1.0-kb *Pst*I fragment from a *Drosophila* hsp70 gene (C) at 42°C in 50% formamide. The size was estimated by comparison to glyoxalated *Hin*dIII-digested DNA of phage lambda. Note the difference in exposure times required for the different hybridization probes: (A) and (B), 1 h; (C), 7 days. The exposure times for the dot blots are indicated.

nitrogen. The dried pellet was extracted three or four times with sterile water, and the extracts were pooled. The RNA was precipitated once more with 0.1 volume of 2 M sodium acetate (pH 5.0) and 2 volumes of 95% ethanol (−20°C). The RNA was centrifuged at 12,000 × *g* (20 min, −10°C), dried under nitrogen, and dissolved in sterile water to a final concentration of 2 mg/ml. Typical yields of total cellular RNA were about 1 mg per five roller bottles of cells. Polyadenylated [poly(A)$^+$] RNA was separated from the total RNA by two passages through an oligodeoxythymidylate-cellulose column (Collaborative Research Inc.) by the procedure of Aviv and Leder (3). All glassware and solutions were treated with 0.1% diethylpyrocarbonate and baked or autoclaved, respectively, before use.

Construction and screening of the cDNA library. Complementary DNA was synthesized by the method of Wickens et al. (45), with minor modifications. The starting material was 5.5 μg of poly(A)$^+$ RNA from arsenite-induced RTG-2 cells. Avian myeloblastosis virus reverse transcriptase (Life Sciences Inc.) was used at a ratio of 11.5 U per μg of poly(A)$^+$ RNA in a reaction volume of 100 μl. After second-strand synthesis with 30 U of *Escherichia coli* DNA polymerase I (New England Nuclear) and S1 nuclease (Bethesda Research Laboratories) digestion, a total of 134 ng of double-stranded, S1-resistant cDNA was recovered. This cDNA was inserted into the *Pst*I site of pBR322 by the dG-dC tailing method of Deng and Wu (8). The annealed DNA was used to transform *E. coli* RR1 cells by the method of Dagert and Ehrlich (7). Transformation efficiency with annealed DNA was 1.5×10^5 CFU/μg, with a background of ampicillin-resistant clones of 10 to 15%.

The cDNA library was screened with a nick translated (32) 1-kilobase (kb) *Pst*I fragment from an hsp70 gene of *D. melanogaster*. The *Drosophila* gene was from clone 132E3, described by Moran et al. (30). The screening of about 700 independent clones yielded two (pTHS70.7 and pTHS70.14) which hybridized to the *Drosophila* hsp70 gene.

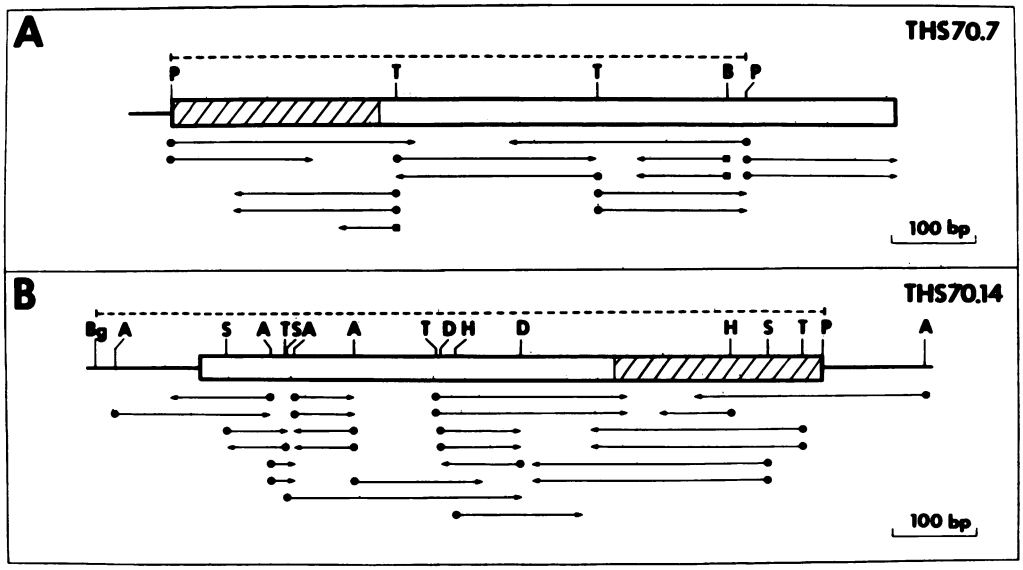

FIG. 2. Partial restriction map and strategy used to determine the nucleotide sequences of THS70.7 (A) and THS70.14 (B) cDNAs. Arrows represent the direction of sequencing from Klenow-labeled fragments, using either the chemical cleavage method (■) or the dideoxy termination method (●). The lengths of the arrows represent the actual number of nucleotides sequenced from each site. The areas used as hybridization probes are indicated by a dashed line above the maps. The boxed regions represent the cDNA sequences, whereas the thin lines represent pBR322 sequences. The hatched area within the boxes indicates the region of overlap between the two cDNAs. The restriction sites are: A, *Ava*II; B, *Bam*HI; Bg, *Bgl*I; D, *Dde*I; H, *Hae*III; P, *Pst*I; S, *Sau*IIIA; T, *Taq*I.

Hybridizations. All hybridizations were carried out at 42°C in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, pH 7.0, and 0.1 mM EDTA)–50% deionized formamide–1× Denhardt reagent (9)–0.1% sodium dodecyl sulfate (SDS)–100 μg of sheared, denatured calf thymus DNA per ml. After hybridization (usually overnight), the filters were washed in two changes of 2× SSPE–0.1% SDS followed by one change of 0.1× SSPE–0.1% SDS, all at room temperature. One final wash was carried out at 50°C in 0.1× SSPE–0.1% SDS. The filters were fluorographed at −70°C with Kodak X-Omat AR film and a Dupont Cronex intensifying screen.

RNA Northern and dot blot analysis. Poly(A)$^+$ RNA was denatured in 1 M glyoxal–10 mM sodium phosphate (pH 7.0) at 50°C for 1 h (27), fractionated by agarose gel electrophoresis, and transferred to nitrocellulose paper (Schleicher & Schuell Co.) through 20× SSPE as described by Thomas (41). For the dot blot analysis, total RNA was placed on nitrocellulose paper and allowed to dry. These filters were then baked at 80°C in vacuo for 2 h.

DNA Southern blot analysis. DNA digested with restriction enzymes was fractionated by agarose gel electrophoresis. Transfer of the DNA to nitrocellulose paper was through 20× SSPE as described by Southern (37). After the transfer, the filter was air dried and baked at 80°C in vacuo for 2 h.

Other procedures. Digestions of DNA with restriction enzymes were according to the instructions of the supplier (Bethesda Research Laboratories). Plasmid DNA was isolated according to the method of Birnboim and Doly (4). Genomic DNA was isolated from trout testes essentially as described by Blin and Stafford (5). Sequencing of DNA was performed by either the chemical cleavage method of Maxam and Gilbert (25) or the dideoxy-M13 method of Messing et al. (28).

RESULTS

Analysis of pTHS70.7 and pTHS70.14. Two trout hsp70 cDNA sequences, pTHS70.7 and pTHS70.14, were isolated from a cDNA library made from arsenite-induced trout cells. The identification of these two clones was based on their homology to a *Drosophila* hsp70 gene (see above). To ensure that pTHS70.7 and pTHS70.14 coded for induced hsp70 species, RNA from control and sodium arsenite-treated RTG-2 cells was subjected to Northern and RNA dot blot analysis (Fig. 1). The induction of hsp70 mRNA (approximate size, 2.2 kb) in the arsenite-induced cells was evident. Control cells contained very little (if any) message for hsp70. A heterologous probe from *D. melanogaster* (Fig. 1C) resulted in a much weaker hybridization compared with that of

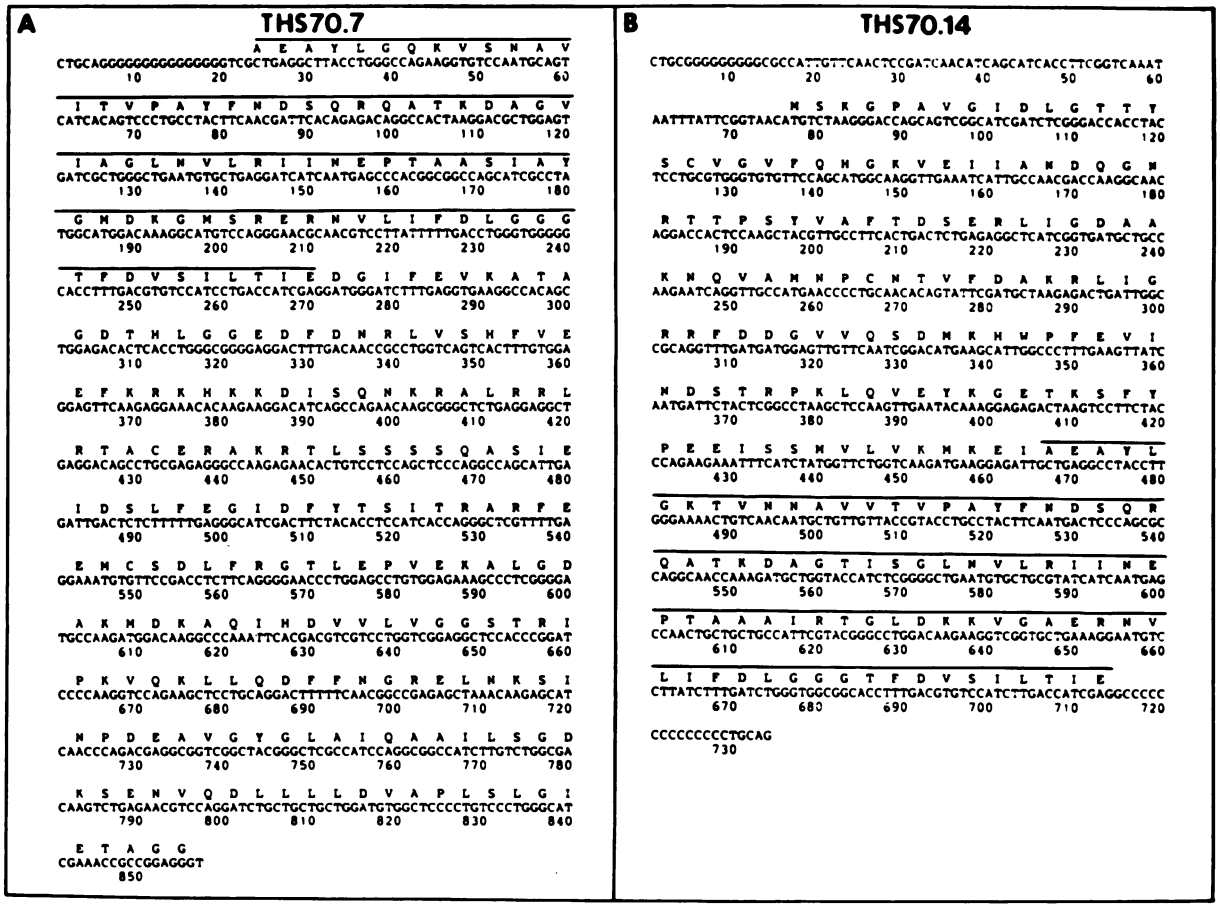

FIG. 3. Nucleotide sequences for THS70.7 (A) and THS70.14 (B) cDNAs with their predicted amino acid sequences. The overlap region between the two different cDNAs is indicated by a line above the respective amino acid sequence. The complete sequence for the hsp70 coding region of THS70.7 is shown. The 3′ end of this cDNA insert (nucleotide 857) was found to be joined to a bacterial insertion element which had become incorporated into this clone. Single-letter amino acid code: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

THS70.7 (Fig. 1A) and THS70.14 (Fig. 1B). This is more obvious when the two sets of RNA dot blots are compared. In this experiment, equal amounts of control and induced RNA were blotted on both filters. The THS70.7 probe gave a much stronger hybridization signal, even at shorter exposure times. Multiple bands in the induced mRNA were revealed by hybridization to labeled THS70.7 (Fig. 1A). This indicated the existence of either an hsp70 multigene family in trout or of spliced intermediates from a single transcript. As will be seen from evidence presented below, the former possibility is more likely.

THS70.7 and THS70.14 were further analyzed by nucleotide sequencing. Partial restriction maps and the sequencing strategy are presented in Fig. 2. The two cDNAs proved to be incomplete copies of hsp70 messages and were from different regions, with an overlap of about 250 nucleotides. The primary nucleotide sequences of THS70.7 and THS70.14 are shown in Fig. 3. The guanine + cytosine contents in the coding regions of both THS70.7 (57.7%) and THS70.14 (50.3%) were relatively high. The overall guanine + cytosine content of the rainbow trout genome has been reported to be 43% (35). Both cDNAs have one long open reading frame (Fig. 3). The THS70.7 sequence contains information for a 278-amino-acid-long region of hsp70. This corresponds to amino acids 128 to 406 of the *Drosophila* hsp70 (13). A serine residue at position 213 of the *Drosophila* hsp70 is deleted from THS70.7. A similar deletion has been reported for the *S. cerevisiae* hsp70 (14). The THS70.14 sequence contains information for the first 213 amino acids of hsp70, assuming that it starts at the first methionine. The predicted amino-terminal sequence for THS70.14 differs from that of the *Drosophila* gene in that it contains an extra three amino acids.

The predicted amino acid sequence of a complete *Drosophila* hsp70 gene and of an inducible *S. cerevisiae* hsp70 gene (YG100) have been published (13, 14). Figure 4 compares a section of these sequences with that of THS70.7. Amino acids that differ are indicated. The highly conserved nature of the three hsp70 sequences is evident. Many of the differences in the three hsp70 sequences occur in the same positions. Conservative amino acid changes account for ca. 55% of the differences between the trout and *Drosophila* sequences and 48% of those between trout and *S. cerevisiae*.

The extent of homology among THS70.7, THS70.14, *Drosophila* hsp70 (13), and YG100 (14) is summarized in Fig. 5. In all cases, the percent homology was calculated for the total sequence information available. As expected, the degree of sequence divergence at the nucleotide level is greater than that at the amino acid level. Interestingly enough, the nucleotide sequences of THS70.7 and THS70.14 are only 73.3% homologous in their overlap region, yet their homology at the amino acid level is quite high (88%). Thus, even within trout, codon preference varies between the two genes in the regions analyzed. Compared with the *Drosophila* hsp70 gene, YG100 has less homology to both THS70.7 and THS70.14 at the nucleotide level. When the amino acid sequences are compared, however, YG100 and the *Drosophila* hsp70 show similar degrees of homology to the trout hsp70 (see Fig. 4).

Detection of multiple hsp70 genes in the trout genome. Genomic DNA was isolated from trout testes and subjected to analysis by the Southern blot technique. Several restriction digests were performed and the DNA was fractionated on agarose gels. After transfer of the DNA to nitrocellulose filters, hybridizations to hsp70 probes were carried out under stringent conditions. The results in Fig. 6A were obtained with a nick-translated THS70.7 fragment, which spans amino acids 128 to 348 of the hsp70 and contains a *Pst*I site at the 3′ end. Strong hybridization to two bands was detected when *Pst*I was used to cleave the genomic DNA (Fig. 6, lane 1), suggesting the presence of at least two hsp70 genes. Hybridization to *Bam*HI-cleaved DNA (Fig. 6, lane 2) revealed a larger fragment of ca. 8 kb. The existence of two hsp70 genes on this *Bam*HI fragment is possible, since the

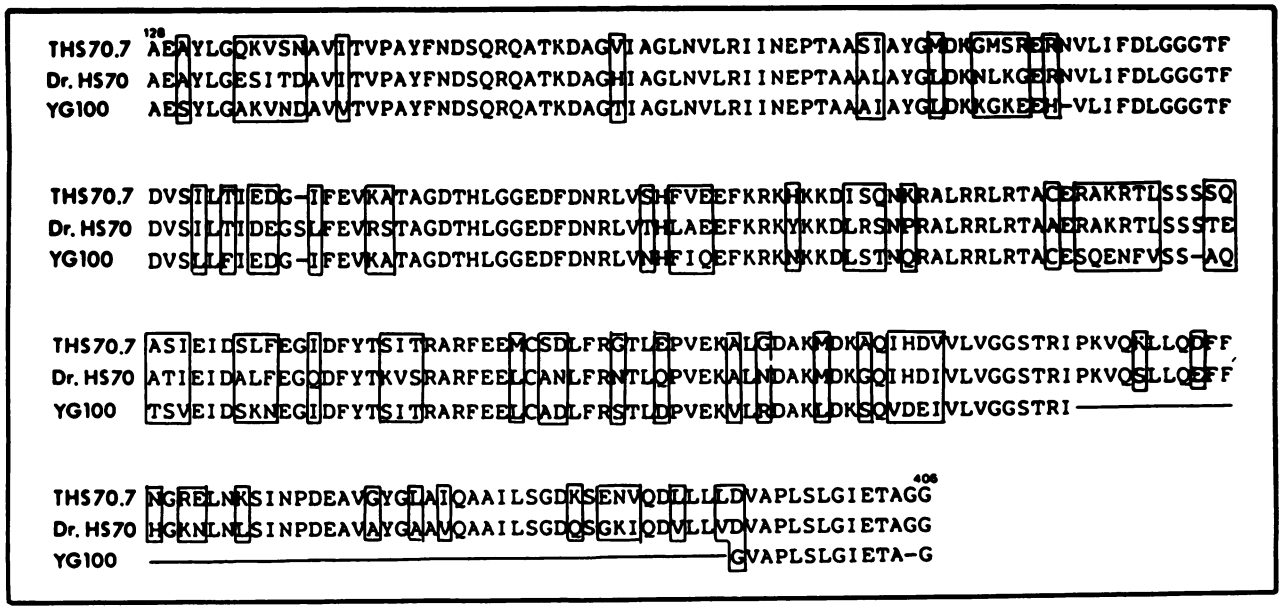

FIG. 4. Comparison of the predicted amino acid sequences of THS70.7, a *Drosophila* hsp70 gene (taken from Ingolia et al. [13]), and *S. cerevisiae* hsp70 gene YG100 (taken from Ingolia et al. [14]). Regions compared are amino acids 128 to 406, based on the *Drosophila* hsp70 numbering. Mismatches in the sequences are boxed in. The single dashes represent deleted amino acids relative to the compared sequence. The solid line in the YG100 sequence represents information not yet available.

	THS70.7	THS70.14	Dr.HS70	YG100
THS70.7	A / B	73.3	72.0	66.2
THS70.14	88.1		71.3	67.5
Dr. HS70	79.1	79.5		64.1
YG100	79.0	77.1	72.0	

FIG. 5. Matrix summary of the nucleotide homology (A) and amino acid homology (B) among THS70.7, THS70.14, *Drosophila* hsp70, and YG100. The homologies are given as percentages of the total available sequence information.

digestion of trout DNA with both *Bam*HI and *Pst*I resulted in the disappearance of the large *Bam*HI fragment and the appearance of the two smaller *Pst*I fragments (Fig. 6, lane 6). However, the existence of two different *Bam*HI fragments, each containing an hsp70 gene, is also a possibility. Hybridization to *Eco*RI-cleaved trout DNA (Fig. 6, lane 3) revealed a number of bands of different intensities, possibly due to incomplete digestion of the DNA. Genomic trout DNA was also subjected to double digests and hybridized to the THS70.7 fragment (Fig. 6, lanes 4 to 6) or to the 1.0-kb *Pst*I fragment from a *Drosophila* hsp70 gene (Fig. 6, lanes 7 and 8) spanning animo acids 1 to 312 in addition to some 5′ noncoding sequence. Both probes produced similar results, but THS70.7 resulted in much better signals, as expected. The detection of two hsp70 sequences in the trout genome does not rule out the presence of additional hsp70 genes. Sequence divergence and the incomplete nature of the cDNA probes used might allow other hsp70 sequences in the genome to go undetected. It is worth noting, however, that hybridizations at lower stringency yielded results similar to those shown here (data not shown).

To investigate interspecies hsp70 sequence homology, we cleaved genomic DNA from a variety of sources (trout testes, RTG-2 cells, HeLa cells, *C. elegans*, bovine liver, and *D. melanogaster*) with *Pst*I and subjected it to Southern blot analysis. Hybridization was to either THS70.14 (Fig. 7, lane 1) or to the THS70.7 fragment described above (Fig. 7, lanes 2 to 7). It should be noted that the filters containing trout DNA were exposed to film for a much shorter time than the filters containing the other DNAs. The THS70.14 fragment (spanning amino acids 1 to 210 and some 5′ noncoding region) hybridized to the same two *Pst*I fragments in trout DNA as those detected by the THS70.7 probe. In addition, two larger *Pst*I fragments were evident, revealing hsp70 sequences that were not detected by the THS70.7 probe. When trout testis DNA was compared with RTG-2 DNA, two common *Pst*I fragments hybridized to the hsp70 probe. The RTG-2 DNA also contained a couple of larger *Pst*I fragments that hybridized to THS70.7. These extra fragments were due to the incomplete nature of the digestion of RTG-2 DNA with *Pst*I. Similar results have been observed in partial *Pst*I digests of trout testis DNA (data not shown). Cross-hybridization of the trout hsp70 sequences with sequences in the genome of other organisms was also evident (Fig. 7, lanes 4 to 7). Multiple bands were evident with HeLa cell DNA, bovine liver DNA, and *D. melanogaster* DNA. Hybridization to the *C. elegans* DNA was very weak and may be due to the relatively high adenine-thymine content (64%) of its genome (38).

DISCUSSION

In the present study we analyzed two hsp70 cDNAs, THS70.7 and THS70.14, from rainbow trout. The predicted amino acid sequences from these cDNAs were very similar to those reported for the hsp70 genes of *D. melanogaster* (13) and *S. cerevisiae* (14). The presence of a multigene family of hsp70 sequences in the trout genome was inferred from the hybridization of genomic DNA blots to the hsp70 cDNAs. The existence of multiple hsp70 genes in trout is also supported by the presence of multiple spots on two-dimensional polyacrylamide gels of protein samples from in vitro-translated, hybrid-selected mRNA for trout hsp70 (E. Burgess, personal communication).

The levels of hsp70 mRNA in sodium arsenite-induced RTG-2 cells are much greater than those in control cells. The kinetics of induction of the hsp70 message are also very rapid, i.e., of the order of minutes (results not shown). The multiple bands observed on RNA Northern blots hybridized

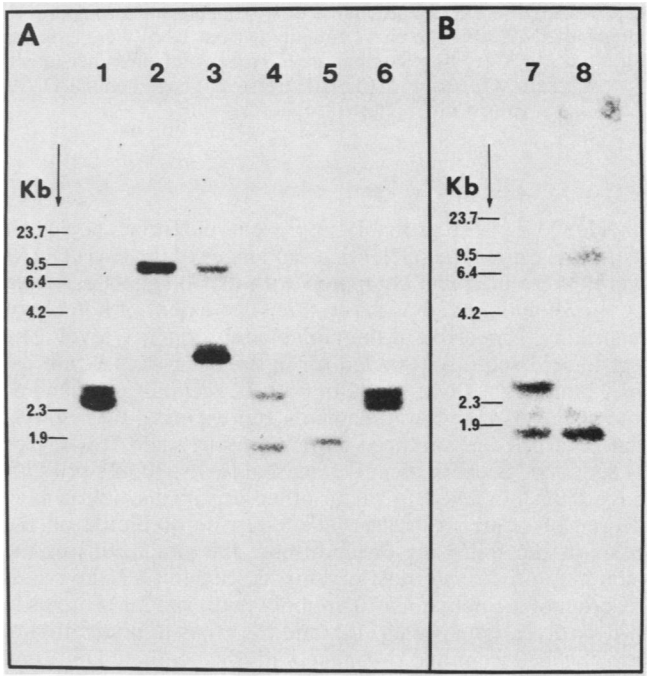

FIG. 6. Southern blot analysis of genomic DNA isolated from trout testes. Approximately 6 μg of the genomic DNA was digested with *Pst*I (lane 1), *Bam*HI (lane 2), *Eco*RI (lane 3), *Pst*I and *Eco*RI (lanes 4 and 7), *Bam*HI and *Eco*RI (lanes 5 and 8), or *Pst*I and *Bam*HI (lane 6) and separated by electrophoresis on a 1.2% (A) or 0.9% (B) agarose gel. The DNA was then transferred to nitrocellulose filters. Hybridization was to ^{32}P-labeled THS70.7 (A) or to a 1.0-kb *Pst*I fragment from a Drosophila hsp70 gene (B) at 42°C in 50% formamide. The size markers are from a *Hin*dIII digest of phage lambda DNA.

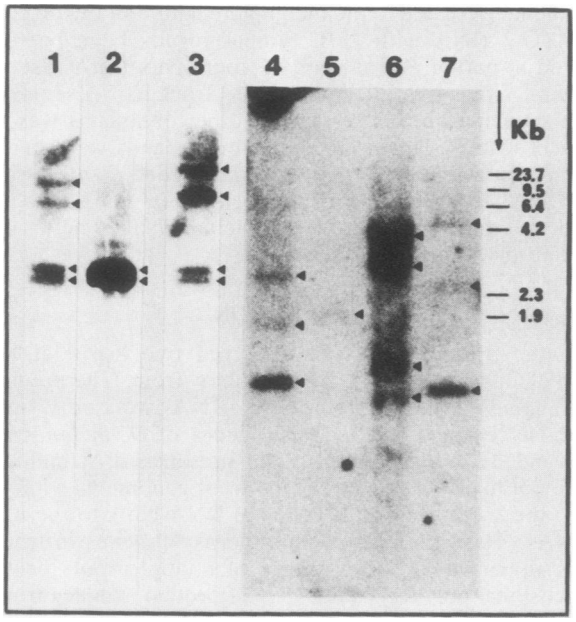

FIG. 7. Southern blot analysis of *Pst*I-digested genomic DNA from various sources: 6 μg of trout testis DNA (lanes 1 and 2), 4 μg of RTG-2 DNA (lane 3), 6 μg of HeLa cell DNA (lane 4), 3 μg of *C. elegans* DNA (lane 5), 8 μg of bovine DNA (lane 6), and 3 μg of *D. melanogaster* DNA (lane 7). The DNA was cleaved, fractionated by electrophoresis through a 1.0% agarose gel, and transferred to nitrocellulose filters. Hybridization was to ^{32}P-labeled THS70.14 (lane 1) or THS70.7 (lanes 2 to 7) at 42°C in 50% formamide. The hsp70-related sequences are indicated by triangles next to appropriate bands. Note that the filters containing trout DNA were exposed for 2 days to X-ray film, compared with 8 days for the other filters. Size markers were from a *Hin*dIII digest of phage lambda DNA. Lane 3 was from a different electrophoresis run.

to THS70.7 most probably represent different species of hsp70 message. The nucleotide sequences of the two cDNAs were determined and compared with hsp70 sequences from *D. melanogaster* and *S. cerevisiae*. The extent of homology is striking, especially at the amino acid sequence level. The amino acid sequences coded for in the trout cDNAs are ca. 79% homologous with those of both *D. melanogaster* and *S. cerevisiae* hsp70. For organisms representing three phyla that diverged early in the evolutionary time scale, this degree of sequence conservatism is remarkable. hsp70-like proteins have been observed in many other organisms, and a high degree of sequence conservatism can be predicted on the basis of the following observations: the similarities of the induction process in most organisms studied (34), the cross-reaction of a chicken hsp70 antibody with similar proteins in widely divergent species (16), and the cross-hybridization of hsp70 genes from one organism with the genomic DNA and RNA of other organisms (10, 14, 24, 29, 36; this report). The conservatism of the heat shock response is not limited to the hsp70-like proteins; similarities among different hsp83-like proteins (16) and among the small hsps (12, 33) have been reported.

Although an exact role for hsp70 has not yet been reported, studies have shown the protein to be present in the nucleic of stressed cells (1, 21, 40, 43, 44). A protective role for hsps in the cell is supported by studies showing that a mild heat shock preceding a normally lethal heat shock confers thermotolerance on cells (23, 26, 31). Such thermotolerance can be conferred upon cells by other inducers of hsps, such as sodium arsenite (23).

Lee et al. (19) have recently suggested that the common factor among inducers of the heat shock response may be the development of an oxidation stress in cells. This intriguing hypothesis could explain the widespread existence of hsps in both procaryotic and eucaryotic cells, since the necessity for protection from the adverse physiological effects of excess intracellular oxygen presumably dates from early evolutionary times.

The results presented here support the conclusion that the hsp70 genes form a highly conservative gene family. Further studies are underway to determine the genomic organization of the trout hsp70 genes.

ACKNOWLEDGMENTS

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