

Construction of cDNA library from liver RNA of heat shocked rats and DNA sequence analysis of the clone containing the 3'-end untranslated region (3'UTR) of the heat inducible gene hsp70.2*

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cDNA library constructed from liver RNA of rats subjected to hyperthermia was used to isolate divergent 3' end untranslated regions (3'UTR) of heat inducible hsp70.1 and hsp70.2 genes. As a result of a double selection procedure with the use of DNA-DNA hybridization and PCR analysis 9 clones containing cDNA sequences derived from the 3'UTR of the hsp70.1 gene and 16 clones containing cDNA sequences derived from the 3'UTR of the hsp70.2 gene were selected. Nucleotide sequence of the cloned inserts was established and the Northern blot analysis was performed to identify the heat inducible transcript encoded by the hsp70.2 gene.

Heat shock or "stress" genes are induced in cells exposed to a variety of environmental factors (reviewed in [1-4]). The most prominent class of the heat shock genes is the hsp70 multigene family. In mammals multiple genes which belong to this family are constitutively expressed in normal physiological conditions (e.g. in rats: the hsc70 gene [5], grp78 gene [6], hst70 [7], hsp70.3 [8], mthsp70 gene [9]), while only two or three genes are heat inducible.

In rat cells heat shock induces synthesis of two hsp70-related transcripts which were supposed to be encoded by two separate genes. Genomic sequences containing two hsp70 transcription units referred to as the hsp70.1 gene [10, 8, 11, 12] and the hsp70.2 gene [8] were recently isolated and sequenced. The DNA sequence in the 5'-end untranslated regions and

the coding region of the two genes is almost identical [8]. Almost perfect homology extends up to 26 bp past the termination codon while the DNA sequences of the downstream 3' UTR of the hsp70.1 and the hsp70.2 genes are divergent [8].

In order to fully characterize the relation between a given gene and the transcript it codes for, as well as to determine the 3' end of the investigated gene it is important to compare the genomic sequences and the corresponding cDNA sequences derived from the transcript. In the case of the above-mentioned heat-inducible hsp70 genes of the rat both genomic and cDNA sequences were known so far only for the hsp70.1 gene [11, 12]. To unequivocally establish the relation between another hsp70 heat-inducible transcript and already known

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Abbreviation: nt, nucleotide(s).

genomic sequences of the hsp70.2 gene we constructed and analyzed a cDNA library from liver RNA of rat subjected to hyperthermia. Clones containing cDNAs corresponding to the 3'-end untranslated region of the hsp70.1 and the hsp70.2 genes were isolated and sequenced.

METHODS

Animals. 2–2.5 month old Wistar rats, starved for 18 h before the experiment were used. The animals were anesthetized by intraperitoneal injection of Vetbutal (50 mg/kg body weight). Rats were heat shocked by immersion in a water bath (3/4 of the body) set at 42°C for 45 min as described earlier [13]. Then the rats were killed by decapitation, livers were immediately excised, frozen in liquid nitrogen and pulverized.

Isolation of RNA. RNA was isolated from 1 g of pulverized liver by the "one step method" of Chomczyński & Sacchi [14]. Poly (A)⁺ RNA was separated by two rounds of affinity chromatography on the oligo dT cellulose column (Boehringer).

Construction and screening of cDNA library. cDNA library was constructed using ZAP-cDNA Gigapack II Gold Cloning Kit (Stratagene) according to the instruction manual. Poly (A)⁺ RNA (5 µg) from the liver of heat shocked rat was used for the cDNA synthesis. cDNA was cloned unidirectionally in Uni-ZAPII vector into the *EcoRI* and *XhoI* restriction sites. First screening was performed as recommended by the above Kit manufacturers. Further selection of the cDNA clones was done by the PCR method.

PCR reactions. The reactions were performed in the total volume of 50 µl, with 2 u of Tag polymerase (Promega) in the buffer supplied by manufacturer and 1 mM MgCl₂. Primers were applied at concentrations of 20 pM each. Samples were denatured in thermal cycler at 94°C for 3 min, amplified by 30 cycles of PCR at 94°C, 58°C, 72°C for 1 min each and elongated at 72°C for 5 min. Primers used to amplify the C-terminal part of the coding region of the hsp70.1 gene were: primer C — 5' AAG CTG GAC AAG GCG CAG 3' complementary to the nt 1633–1651 of the hsp70.1 gene sequence [10], and primer F — 5' CAA CAC CCT GAG AGC CAG 3' complementary to the nt 2594–2612 of

the above sequence. Primers complementary to the 3'UTR of the rat hsp70.1 gene were: primer E — 5' CTG GCT CTC AGG GTG TTG 3', complementary to the nt 2594–2612 of the hsp70.1 gene sequence [10], primer H — 5' ACA GGG TGG CAG TGC TGA 3', complementary to the nt 2836–2855 of the above sequence. Primers complementary to the 3'UTR of the rat hsp70.2 cDNA were: PS HSP70.2 — 5' CTC CTT CGT TCG GTG TGC 3', complementary to the nt 124–141 of the cDNA sequence contained in the #5 clone (nt 2011–2029 in the genomic sequence published by Walter *et al.* [8]) and MS HSP70.2 — 5' GCC AGG CAA GAT TAT AT 3' complementary to the nt 546–573 of the #5 cDNA clone sequence (nt 2435–2452 in the genomic sequence).

Sequencing of DNA. Sequencing was performed with Polymerase T7 Kit (Pharmacia) as described earlier [10]. Nucleotide sequence was analysed using PC/Gene 11.0 (IntelliGenetics Inc.) software. Computed sequence alignments were later precisely adjusted manually.

Northern blot analysis. Poly(A)⁺ RNA was separated in 1.2% agarose gel containing 2.2 M formaldehyde (in 1 × Mops buffer), blotted onto Hybond-N membrane and fixed at 80°C for 1.5 h. Hybridization was carried out at 58°C in a mixture containing 50% formamide, 3 × SSC, 5 × Denhardt's solution, 1% SDS, 100 µg/ml salmon sperm DNA and radioactive probe as described earlier [10]. Probes were labeled with [³²P]dCTP using Random Primer Kit (Amersham).

RESULTS AND DISCUSSION

About 10⁶ plaques were screened using the probe A, containing sequences from C-terminal part of the coding unit of the rat heat inducible hsp70.1 gene cloned by us previously [10]. As we were looking for the clones containing the sequences of the 3'UTR of the hsp70 genes this probe allowed to detect relatively short inserts. Also, due to cross-hybridization, clones originating from transcripts of both hsp70.1 and hsp70.2 genes could be selected. Out of about 100 positive plaques 25 large ones, well separated from each other, were selected for further analysis. About 1 cm² of agar layer surrounding each plaque was picked up and allowed to diffuse in 1 ml of SM buffer [15] containing 20

cDNA HSP70.2	GCTCTGGGT CGGGGCC ACCATCGAGGAGGTGGATTAGAGGCTCTTTCTGGCGCT	1942
cDNA HSP70.2	CCAGGTGTGATCTAGGAGACAGATGGGTGGCCTTGAGGACTTTGGGTTATTGTGC	1997
cDNA HSP70.2	TTTAGGACATTA ACTCCTTCGTTCCGGTCTGCAATCAAGTCCTAGGTTTAAGCAAA	2052
cDNA HSP70.2	CTGCCTTCCATTTACTCTGTGGAATTTACCGTGTG-CTTGCATTCCCAGTAAATA..-.....A.....	2106
cDNA HSP70.2	TAGTACTGGGAGTGTGTCTTTGCAATAGATATAATTTCTGCCTTCAAGTCAGCA	2161
cDNA HSP70.2	CTGCCCCCCCCCGAAGTTATTTCTTTTG-CAGGACAGTCAGAGCTATATTGATAA....CAC.....	2216
cDNA HSP70.2	TAGCAAGAGGTGTGTTACAAAAACACCAGGACACTGTTGAGTTCCTTTGTGTTTG --.....GA.....	2269
cDNA HSP70.2	GACTCTCCCCTGGGCGACAGTGTGAGGCACTGTTAAGTCAGGAGCTC-GGGGCCA.....	2324
cDNA HSP70.2	ACCGGTGGAT-CACTGAAAGCTGAGACTCTGTTGCTTCTCCCGTTTGACACTCTGT.....	2379
cDNA HSP70.2	TTGCTTTCCTTGCATGGTGGCTCACCTAAGGCTGAGACTCTGTTCTCCTTCCCT	2434
cDNA HSP70.2	ATATAATCTTGCCTGGC-GTTGCACTTGTTCCCCAGTGTGTGAACTCGGAGATGAC.....	2489
cDNA HSP70.2	GTTTACACCACCACTGTTAGTTCACGTTTTTTGTTTTTACATAACCATCCTGAAC	2544
cDNA HSP70.2	TCAGGTCAATTTTTAGCTGGCTATTTGAAATAAACTTCAAAA-GAACTTGCCA(A)²⁷A.....	2599

Fig. 1. Alignment of the cDNA sequence cloned in #5 phagemid with the corresponding sequence of the rat *hsp70.2* gene [8].

Identical residues are indicated by a dot, gaps inserted to maximize the identity are indicated by a hyphen. The coding sequence is shown in bold print, polyadenylation signals are double underlined, primer sequences are underlined.

μ l of chloroform. From each of above phage solutions 1 μ l was taken for the PCR reaction with the primers complementary to the 3'UTR of the rat *hsp70.1* gene. This approach allowed us to select the clones containing cDNA of the *hsp70.1* gene. Nine positive clones were detected. Two of them as well as two negative clones were submitted to the "in vivo excision" procedure of the pBluescript SK(-) phagemid from the Uni-Zap XR vector performed in *E. coli* cells (SOLR strain). Excision of two positive and only one negative clone was successful. Inserts of the rescued phagemids (positive clone #8 and the negative clone #5) were sequenced and compared to the known genomic sequences of the rat *hsp70.1* and *hsp70.2* genes. The insert of the clone #8 was shown to be 1053 bp long and contained a sequence of 259 C-termi-

nal codons, complete 3'UTR of the *hsp70.1* gene and (A)₁₈ tract. Transcript of the gene ended 11 nucleotides past the polyadenylation signal.

The insert cloned in the #5 phagemid was 741 bp long and was in 98.5% homologous to the corresponding 3' end part of the rat *hsp 70.2* gene cloned by Walter *et al.* [8]. It contained a sequence of 13 C-terminal codons, 668 bp of the 3'UTR of the gene and (A)₂₇ tract. Within the cDNA sequence two polyadenylation signals were detected (Fig. 1) in agreement with the data obtained for the genomic DNA sequence [8]. The transcript of the *hsp70.2* gene ended 18 nucleotides past the distal polyadenylation signal (AATAAA, underlined; Fig. 1).

Sixteen *hsp70.1*-negative clones were checked for the presence of the *hsp70.2* cDNA sequences by the PCR reaction with the use of primers

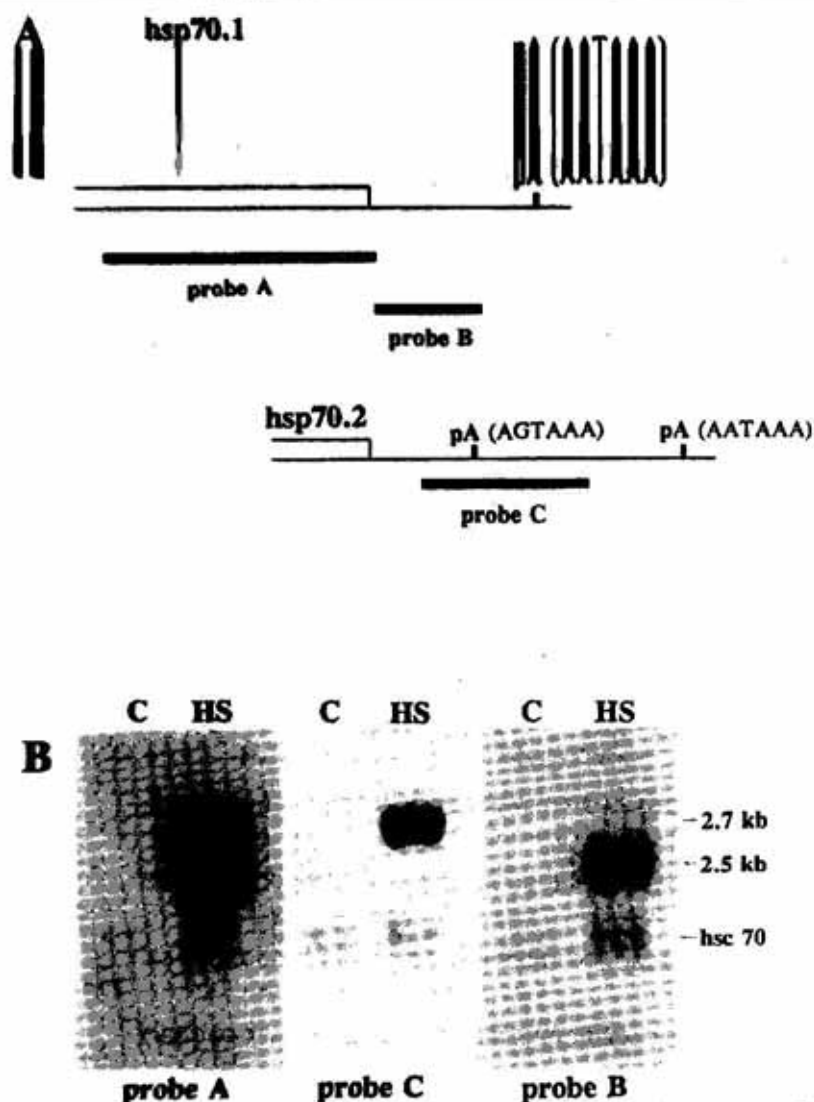


Fig. 2. (A) Schematic representation of the sequences used to generate probes.

Probe A, containing the C-terminal part of the hsp70.1 gene coding unit, was amplified from the rat hsp70.1 gene DNA using primers C and F (see Methods). Probe B — corresponding to the 3'UTR of the hsp70.1 cDNA was amplified from the #8 cDNA clone with the primers E and H. Probe C corresponding to the 3'UTR of the hsp70.2 cDNA was amplified from the #5 cDNA clone with the PS hsp70.2 and MS hsp70.2 primers. Open box represents the protein coding sequences, pA indicates the polyadenylation signal.

(B) Northern blot analysis of two hsp70 genes expression in liver of rats subjected to hyperthermia.

Each lane was loaded with 5 µg of poly (A)⁺ RNA. Blots were hybridized with the probe A, B or C, respectively, then the probe was stripped off and blots were rehybridized with the probe complementary to the constitutive hsc70 gene sequences.

complementary to the sequence from #5 clone (see Methods). Primers enabled amplification of the 441 bp fragment of the hsp70.2 3'UTR encompassing the alleged imperfect proximal polyadenylation signal (AGTAAA, underlined; Fig. 1). All analyzed clones were hsp70.2-positive, and originated from the transcripts which arose with omission of the proximal polyadenylation signal; this confirmed that the latter is not functional or rarely used.

In tissues of rat subjected to whole body hyperthermia two transcripts of 2.5 kb and 2.7 kb are synthesized from heat-induced hsp70 genes (Fig. 2b). We have recently shown that the 2.5 kb transcript is encoded by the hsp70.1 gene [10] (see also Fig. 2b). The data from the cloning of genomic sequences [8] suggested that the 2.7 kb transcript could originate from transcription of the hsp70.2 gene. Figure 2b shows that isolated by us cDNA fragment derived from the region corresponding to 3'UTR of the hsp70.2 gene, does, in fact, specifically hybridize with the 2.7 kb transcript.

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