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The human heat-shock protein family

Expression of a novel heat-inducible HSP70 (HSP70B′) and isolation of its cDNA and genomic DNA

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The human heat-shock protein multigene family comprises several highly conserved proteins with structural and functional properties in common, but which vary in the extent of their inducibility in response to metabolic stress. We have isolated and characterized a novel human HSP70 cDNA, HSP70B′ cDNA, and its corresponding gene sequence. HSP70B′ cDNA hybrid-selected an mRNA encoding a more basic 70 kDa heat-shock protein than both the major stress-inducible HSP70 and constitutively expressed HSC70 heat-shock proteins, which in common with other heat-shock 70 kDa proteins bound ATP. The complete HSP70B′ gene was sequenced and, like the major inducible HSP70 gene, is devoid of introns. The HSP70B′ gene has 77% sequence similarity to the HSP70 gene and 70% similarity to HSC70 cDNA, with greatest sequence divergence towards the 3′-terminus. The HSP70B′ gene represents a functional gene, as indicated by Northern-blot analysis with specific oligonucleotides, hybrid-selected translation with a specific 3′ cDNA sequence and S1 nuclease protection experiments. In contrast with HSP70 mRNA, which is present at low concentrations in HeLa cells and readily induced by heat or $CdCl_2$ treatment in both fibroblasts and HeLa cells, HSP70B′ mRNA was induced only at higher temperature and showed no basal expression. The differences in patterns of induction may be due to the special features of the promoter region of the HSP70B′ gene.

INTRODUCTION

The response of eukaryotic cells to elevated temperature and other forms of metabolic stress involves the expression of a set of heat-shock proteins. The major stress-inducible protein, HSP70 (Wu *et al.*, 1985; Hunt & Morimoto, 1985), is one of a group of related proteins of approx. 70 kDa that is highly conserved throughout evolution and constitutes a multigene family (Lindquist, 1986; Lindquist & Craig, 1988). Other related proteins differ in the extent of their stress-inducibility and in their subcellular location. These include the cognate HSC70 protein (O'Malley *et al.*, 1985; Sorger & Pelham, 1987; Dworniczak & Mirault, 1987), which is constitutively synthesized in a wide variety of cell types, and the larger glucose-regulated endoplasmic-reticulum protein GRP 78 (Munro & Pelham, 1986). There are also reports of different isoforms of 70 kDa heat-shock proteins (Whatley *et al.*, 1986; Napolitano *et al.*, 1987), some of which may arise through post-translational modification.

In the evolutionarily conserved heat-shock protein family there is extensive sequence homology between the related 70 kDa heat-shock proteins. We have previously reported the hybrid-selected translation of rat brain HSC70 mRNA by use of the *Drosophila* HSP70 gene under conditions of high stringency (Lim *et al.*, 1984). To distinguish closely related human (and rat) 70 kDa heat-shock mRNA species, we have utilized specific 3′ cDNA sequences under high-stringency hybridization conditions. In the present paper we describe the isolation and expression of a new human stress-inducible HSP70 gene (HSP70B′ gene) encoding a more basic 70 kDa protein, and its induction characteristics in response to conditions of heat stress and $CdCl_2$ treatment. The HSP70B′ gene is most closely related to (but not identical with) a previously isolated 5′ HSP70 genomic fragment (Voellmy *et al.*, 1985), which has not yet been definitively shown to be part of an expressed HSP70 gene. We have designated the new gene sequence HSP70B′ to distinguish it from the 5′ segment, which has been referred to as HSP70B (Schiller *et al.*, 1988).

MATERIALS AND METHODS

Materials

ATP–agarose was from Sigma Chemical Co. (Poole, Dorset, U.K.). All radiochemicals were from Amersham International (Amersham, Bucks., U.K.). The human λ 2001 genomic library was from Dr. T. H. Rabbitts (M.R.C. Laboratory of Molecular Biology, Cambridge, U.K.) and all other materials and cell lines as stated in previous publications (Whatley *et al.*, 1986; Hall *et al.*, 1987; Goate *et al.*, 1987).

Isolation and DNA sequence analysis of cDNA and genomic clones

cDNA copies of heat-shocked human fibroblast polyadenylated RNA were synthesized and cloned in plasmid pBR322 as previously described (Hall *et al.*, 1987). Rat brain cDNA clones (10^4) (Hall *et al.*, 1987) were screened with a ^{32}P-labelled HSP70 gene probe (pH 2.3; Wu *et al.*, 1985). Several clones corresponding to the rat HSC70 cDNA as determined by sequence analysis (C. Monfries & T. K. C. Leung, unpublished work; O'Malley *et al.*, 1985) were isolated. Similarly human fibroblast cDNA clones (10^4) were screened in duplicate with pH 2.3 (HSP70 cDNA) and with a rat HSC70 cDNA (JM33; 1.4 kb). Positive clones of three different types (on the basis of their

Abbreviations used: HSP70, 70 kDa heat-shock protein; HSC70, 70 kDa heat-shock cognate protein.
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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases.

hybridization to the two probes) were isolated and identified by DNA sequence analysis as cDNA for the human HSC70 (e.g. WS4; 700 bp), the human major HSP70 (e.g. WS20; 650 bp) and a novel inducible HSP70 (e.g. WS8; 1 kb). All colony hybridizations (Grunstein & Hogness, 1975) were performed at 60 °C in medium containing 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, 100 μg of denatured salmon testis DNA/ml and 10 μg of poly(A)/ml. Filters were washed at 62 °C in medium containing 2 × SSC and 0.1% SDS and at 50 °C in medium containing 0.2 × SSC and 0.1% SDS. (1 × SSC is 0.15 M-NaCl/15 mM-sodium citrate buffer, pH 7; 1 × Denhardt's solution is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA.)

A human λ 2001 genomic library (Lefranc *et al.*, 1986) was screened (5×10^5 clones) with a short 3′ fragment of WS8 cDNA (WS8.2.3; 600 bp), and three positive genomic clones analysed by restriction mapping were found to contain the corresponding sequence. cDNA and genomic DNA restriction fragments were subcloned into the M13mp10/M13mp11 vectors, and DNA sequencing was performed by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977), fully covering the two complementary strands for all sequences.

Construction of pHSP70–CAT and pHSP70B′–CAT vectors

A 5′-upstream sequence (500 bp *Bam*HI/*Hin*dIII fragment; Fig. 2*b*) of HSP70B′ genomic DNA was inserted into the plasmid pBLCAT (Luckow & Schütz, 1987) to give the pHSP70B′–CAT construct. Similarly a 1.3 kb *Bam*HI/*Hin*dIII fragment of HSP70 genomic DNA (Wu *et al.*, 1985; Fig. 2*b*) was used to produce pHSP70–CAT. Plasmids purified by the CsCl-density-centrifugation method (Maniatis *et al.*, 1982) were used for transfection experiments.

Cell culture and transfection

Human fibroblasts and HeLa cells were cultured in Eagle's minimal essential medium containing 10% (v/v) foetal-calf serum in an atmosphere of 5% CO_2 in air. At confluency cells were heat-shocked at the described temperature (usually 45 °C) for 20 min, recovered for 2 h at 37 °C and either pulse-labelled with [^{35}S]methionine (Whatley *et al.*, 1986) for 1 h before protein analysis or harvested for RNA preparation by using a guanidinium thiocyanate method involving CsCl density centrifugation (Kaplan *et al.*, 1979; Whatley *et al.*, 1984). Polyadenylated RNA was isolated by oligo(dT)–cellulose chromatography (Hall *et al.*, 1984). For certain experiments confluent fibroblasts were treated with $CdCl_2$ (100 μM) for 1 h, before 1 h recovery, harvesting and preparation of RNA.

For transfection experiments, HeLa cells at 70% confluency were harvested and electroporated (by using a Bio-Rad Gene Pulsar at setting of 250 V/960 μF) with either pHSP70–CAT or pHSP70B′–CAT constructs. Cells were plated out and cultured for 40 h before heat shock at various temperatures. Preparation of cell extracts and the chloramphenicol acetyltransferase (CAT) assay were performed as described by Gorman *et al.* (1982).

Northern-blot analysis of RNA and protein analysis

Hybrid-selected translation and Northern-blot analysis of RNA was as previously described (Lim *et al.*, 1984; Hall *et al.*, 1987), with hybridization at 45 °C for 6 h and washing at 60 °C in medium containing 0.1 × SSC and 0.1% SDS. For Northern-blot analysis with sequence-specific oligomers, probes were synthesized for the HSP70B′ DNA (complementary to positions 562–584 in Fig. 2*a*; 5′-CGAGTCATTGAAATAGGCGGGCA-3′) and the HSP70B DNA complementary to positions 562–584 of the Voellmy *et al.* (1985) sequence; 5′-CGAGTTACTGAAA-TAGGTGGGCA-3′) and 5′-end-labelled (specific radioactivity > 10^8 c.p.m./μg) with T4 polynucleotide kinase and [γ-^{32}P]ATP (10 Ci/mmol; Amersham International). HSP70B′ DNA and HSP70B DNA oligonucleotides differed from each other by three nucleotide residues (and from the corresponding region of HSP70 DNA by three and six nucleotide residues respectively). Specificity and the conditions for hybridization were determined by hybridizing to specific DNA sequences. Blots containing 50 μg of control RNA and heat-shock protein RNA were then hybridized with the labelled oligomers at 55 °C with medium containing 6 × SSPE, 5 × Denhardt's solution, 15% (v/v) formamide, 0.1% SDS and 250 μg of salmon testis DNA/ml for 16 h. (1 × SSPE is 0.18 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA.) Blots were washed with three changes of 3 × SSC at 45 °C and autoradiographed for 24–48 h.

ATP–agarose chromatography of fibroblast heat-shock proteins and of hybrid-selected tranlsation products was as described by Welch & Feramisco (1985). One-dimensional SDS/PAGE and two-dimensional gel electrophoresis were performed as described previously (Whatley *et al.*, 1986). Protein determinations were carried out with the Bio-Rad protein kit.

S1 nuclease mapping

For S1 nuclease analysis, a 270 bp *Sma*I–*Sst*I fragment of HSP70B′ DNA (Fig. 2*b*) was isolated and 5′-end-labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP. After denaturation, the probe was hybridized with either 50 μg of control RNA or heat-shock protein RNA from HeLa cells in medium containing 80% (v/v) formamide, 40 mM-Pipes buffer, pH 6.3, 1 mM-EDTA and 0.4 M-NaCl at 41 °C for 16 h in a final volume of 30 μl. S1 nuclease (2000 units; Amersham International) digestion was

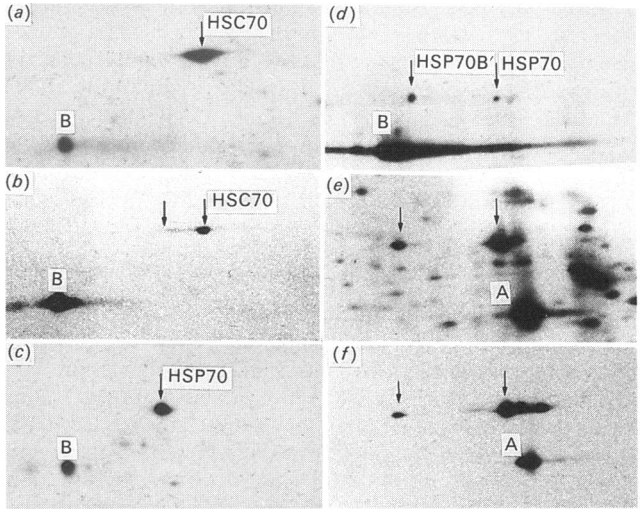

Fig. 1. Hybrid-selected translation and ATP–agarose chromatography of heat-shock proteins

(*a*)–(*d*) Two-dimensional gel-electrophoretic analyses (Whatley *et al.*, 1986) of [^{35}S]methionine-labelled products of hybrid-selected translation: (*a*) rat HSC70 cDNA (clone JM33) hybrid-selected translation of mRNA from rat brain, (*b*) HSC70 cDNA (clone WS4) and (*c*) HSP70 cDNA (clone WS20) hybrid-selected translation of mRNA from human brain, and (*d*) HSP70B′ cDNA (clone WS8) hybrid-selected translation of heat-shocked human fibroblast mRNA. RNA was prepared from rat and human tissues or fibroblasts heat-stressed at 45 °C. B, Reticulocyte-lysate background protein. A two-dimensional gel-electrophoretic analysis of [^{35}S]methionine-labelled heat-shock proteins from fibroblasts heat-shocked at 45 °C is shown in (*e*), and heat-shock proteins purified by ATP–agarose chromatography (Welch & Feramisco, 1985) are shown in (*f*). A, Actin.

carried out in S1 nuclease buffer (containing 280 mM-NaCl, 50 mM-sodium acetate buffer, pH 4.6, 4.5 mM-$ZnSO_4$ and 20 μg of calf thymus DNA/ml) in a final volume of 200 μl for 30 min at 37 °C. After phenol/chloroform extraction and ethanol precipitation, the pellet was dissolved in a loading buffer and electrophoresed on a sequencing gel together with DNA size markers (GIBCO/BRL, Paisley, Renfrewshire, Scotland, U.K.) and sequencing reaction products. The gel was exposed to X-ray film for 5–6 h.

RESULTS

Identification of HSP70B′ cDNA

By using specific 3′ cDNAs, isolated as described in the Materials and methods section, under conditions of high stringency it was possible to specifically hybrid-select heat-shock protein mRNA species and identify their corresponding protein translation products synthesized in reticulocyte lysates by means of two-dimensional gel electrophoresis (Fig. 1). Both rat (Fig. 1*a*) and human (Fig. 1*b*) HSC70 cDNAs hybrid-selected the constitutive HSC70 mRNA from rat and human brain polyadenylated RNA respectively. The human HSP70 cDNA (WS20) hybrid-selected human brain HSP70 mRNA (which is a component of human post-mortem brain mRNA but not of rat brain mRNA; Fig. 1*c*). A third human cDNA sequence (WS8) hybrid-selected mRNA encoding a basic form of heat-inducible 70 kDa protein (designated HSP70B′) from heat-shocked fibroblast mRNA (Fig. 1*d*). A small amount of the highly abundant HSP70 mRNA also cross-hybridized in this instance (cf. Figs. 1*c* and 1*d*). The more basic HSP70 protein (HSP70B′) was also synthesized in addition to the major HSP70 species in confluent human fibroblast cultures heat-shocked at 45 °C (Fig. 1*e*). All three [^{35}S]methionine-labelled 70 kDa proteins bound ATP (Fig. 1*f*), as has previously been demonstrated for heat-shock proteins (Welch & Feramisco, 1985).

Sequence of HSP70B′ cDNA and genomic DNA

The identities of the isolated rat and human HSC70 cDNAs (JM33 and WS4) and human HSP70 cDNA (WS20) were confirmed by DNA sequence analysis and corresponded exactly to published sequences [O'Malley *et al.* (1985), Dworniczak & Mirault (1987) and Hunt & Morimoto (1985) respectively]. HSP70B′ cDNA contained a 1.2 kb 3′ sequence terminating in poly(A) and has 70% identity with the relevant region of HSP70 cDNA (Hunt & Morimoto, 1985). A short 3′ fragment of this cDNA was used to isolate the corresponding HSP70B′ genomic sequence (see the Materials and methods section). Its DNA sequence is shown in Fig. 2(*a*) and was identical with that of the cDNA over the corresponding 1.2 kb region (Figs. 2*a* and 2*b*). HSP70B′ genomic sequence has greatest similarity (94%) to a 5′ truncated HSP70 genomic DNA (Voellmy *et al.*, 1985). The HSP70B′ genomic sequence contains a continuous open reading frame extending from nucleotide residue 120 to 2047. Like the major stress-inducible HSP70 gene, it lacks introns. There is a high level of similarity (77% and 70% respectively) to the human major HSP70 gene (Hunt & Morimoto, 1985) and human HSC70 exon sequences (Dworniczak & Mirault, 1987). The greatest sequence similarity of the HSP70B′ gene to the HSP70 gene is towards the 5′ end, with increasing divergence towards the 3′ end.

This similarity is also reflected in the protein sequence. A comparison of the predicted protein sequences of HSP70B′, HSP70 and HSC70, as well as the truncated *N*-terminal Voellmy *et al.* (1985) sequence, are shown in Fig. 3. The greatest sequence divergence of HSP70B′ is in the *C*-terminal 100 amino acid residues. HSP70B′ protein sequence (643 amino acid residues) has a slightly larger predicted molecular mass (70860 Da) and more basic pI (5.75) than HSP70 (molecular mass 69875 Da; pI 5.37).

The 3′ untranslated region of HSP70B′ DNA, which differs considerably from that of HSP70 DNA, contains a polyadenylation signal 120 nucleotide residues before the poly(A) tract. A second potential polyadenylation signal, AATATA, which is a minor functional hexamer (Birnstiel *et al.*, 1985), is located 26 nucleotide residues upstream of the poly(A) sequence. The promoter region of HSP70B′ DNA also has special features. Although it contains a heat-shock consensus element (from −72; Fig. 2*c*) with multiple GAA/TCC repeats (Amin *et al.*, 1988), it lacks typical TATA and CAAT box sequences, as does the Voellmy *et al.* (1985) 5′ genomic DNA homologue. A comparison of the 5′ promoter regions of HSP70B′ DNA and HSP70B DNA (Voellmy *et al.*, 1985; Schiller *et al.*, 1988) is shown in Fig. 2(*c*). The TATA box region of HSP70B′ DNA differs by one nucleotide residue from that of HSP70B DNA; a GATA box is present at nucleotide residue −30 only in HSP70B′ DNA. There is also a significant difference around the heat-shock consensus element (Fig. 2*c*). HSP70B′ DNA contains a 20-nucleotide-residue insertion immediately after a 10-mer repeat and within the heat-shock element of HSP70B DNA (Schiller *et al.*, 1988). The inserted sequence contains two extra partial repeats and includes a heat-shock-element consensus sequence (Fig. 2*c*).

HSP70B′ gene is expressed and contains a functional heat-inducible promoter

Since there are multiple copies of genes and pseudogenes in the 70 kDa heat-shock protein family (Mues *et al.*, 1986; Harrison *et al.*, 1987; Goate *et al.*, 1987) and there appears to be more that one copy of the HSP70B gene in the human genome, it was important to consider whether the HSP70B′ gene sequence isolated was potentially functional. By using a unique 5′ *Sma*I–*Sst*I fragment of HSP70B′ DNA (Fig. 2*b*), a 135 bp fragment was protected from S1 nuclease digestion by heat-shocked HeLa-cell mRNA (Fig. 4*a*). This enabled the mRNA start site to be determined (see Fig. 2*b*). A basic HSP70 was synthesized only in response to heat-shocked fibroblast mRNA hybrid-selected with HSP70B′ cDNA (Fig. 1*d*) and was the sole product of mRNA selected by a specific 3′ HSP70B′ cDNA sequence (Fig. 4*b*). Furthermore, with specific oligonucleotides corresponding to a region with significant sequence divergence between the HSP70B DNA (nucleotide residues 562–584; Voellmy *et al.*, 1985) and the HSP70B′ DNA (nucleotide residues 562–584; Fig. 2) hybridization to the 2.3 kb heat-inducible mRNA occurred only with the oligonucleotide derived from HSP70B′ DNA, and not that from HSP70B DNA (Fig. 4*c*). Taken together, these results, and the isolation of two cDNAs identical with the HSP70B′ gene sequence over approx. 1.2 kb, strongly suggest that the HSP70B′ DNA sequence isolated represents a functional gene.

The promoter activity of the 5′ region of HSP70B′ DNA was investigated in HeLa cells transiently transfected with either a pHSP70B′–CAT construct (see the Materials and methods section) or a similar construct containing the HSP70 DNA promoter region (Hunt & Morimoto, 1985). Heat-shock of the transfected HeLa cells resulted in induction of chloramphenicol acetyltransferase activity in both cases (Fig. 5), although the basal activity in cells transfected with the HSP70 DNA construct was much greater. The induction profiles clearly demonstrated a functional difference in the two promoter regions. [High basal levels of HSP70 expression have previously been observed in HeLa cells (Whatley *et al.*, 1986; Watowich & Morimoto, 1988).]

(*a*)

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                -131 CCCGGGCGGGCGAGAGGCTCTCAACTGGGCGGGAAGGTGCGGGAAGGTGCGGAAAGGTTCGCGAAAGTTC
-61  GCGGCGGCGGGGGTCGGGTGAGGCGCAAAAGGATAAAAAGCCCGTGGAAGCGGAGCTGAGCAGATCCGAGCCGGGCTGGCTGCAGAGAAA
30  CCGCAGGGAGAGCCTCACTGCTGAGCGCCCCTCGACGGCGGACGGGCAGCAGCCTCCGTGGCCTCCAGCATCCGACAAGAAGCTTCAGCC
120 ATGCAGGCCCCACGGGAGCTCGCGGTGGGCATCGACCTGGGCACCACCTACTCGTGCGTGGGCGTGTTTCAGCAGGGCCGCGTGGAGATC
     1m q a p r e l a v g i d l g t t y s c v g v f q q g r v e i30
210 CTGGCCAACGACCAGGGCAACCGCACCACGCCCAGCTACGTGGCCTTCACCGACACCGAGCGGCTGGTCGGGGACGCGGCCAAGAGCCAG
     l a n d q g n r t t p s y v a f t d t e r l v g d a a k s q60
300 GCGGCCCTGAACCCCCACAACACCGTGTTCGATGCCAAGCGGCTGATCGGGCGCAAGTTCGCGGACACCACGGTGCAGTCGGACATGAAG
     a a l n p h n t v f d a k r l i g r k f a d t t v q s d m k90
390 CACTGGCCCTTCCGGGTGGTGAGCGAGGGCGGCAAGCCCAAGGTGCCGGTATCGTACCGCGGGGAGGACAAGACGTTCTACCCCGAGGAG
     h w p f r v v s e g g k p k v p v s y r g e d k t f y p e e120
480 ATCTCGTCCATGGTGCTGAGCAAGATGAAGGAGACGGCCGAGGCGTACCTGGGCCAGCCCGTGAAGCACGCAGTGATCACCGTGCCCGCC
     i s s m v l s k m k e t a e a y l g q p v k h a v i t v p a150
570 TATTTCAATGACTCGCAGCGCCAGGCCACCAAGGACGCGGGGGCCATCGCGGGGCTCAACGTGTTGCGGATCATCAATGAGCCCACGGCA
     y f n d s q r q a t k d a g a i a g l n v l r i i n e p t a180
660 GCTGCCATCGCCTATGGGCTGGACCGGCGGGGCGCGGGAGAGCGCAACGTGCTCATTTTTGACCTGGGTGGGGGCACCTTCGATGTGTCG
     a a i a y g l d r r g a g e r n v l i f d l g g g t f d v s210
750 GTTCTCTCCATTGACGCTGGTGTCTTTGAGGTGAAAGCCACTGCTGGAGATACCCACCTGGGAGGAGAGGACTTCGACAACCGGCTCGTG
     v l s i d a g v f e v k a t a g d t h l g g e d f d n r l v240
840 AACCACTTCATGGAAGAATTCCGGCGGAAGCATGGGAAGGACCTGAGCGGGAACAAGCGTGCCCTCGGCAGGCTGCGCACAGCCTGTGAG
     n h f m e e f r r k h g k d l s g n k r a l g r l r t a c e270
930 CGCGCCAAGCGCACCCTGTCCTCCAGCACCCAGGCCACCCTGGAGATAGACTCCCTGTTCGAGGGCGTGGACTTCTACACGTCCATCACT
     r a k r t l s s s t q a t l e i d s l f e g v d f y t s i t300
1020 CGTGCCCGCTTTGAGGAACTGTGCTCAGACCTCTTCCGCAGCACCCTGGAGCCGGTGGAGAAGGCCCTGCGGGATGCCAAGCTGGACAAG
     r a r f e e l c s d l f r s t l e p v e k a l r d a k l d k330
1110 GCCCAGATTCATGACGTCGTCCTGGTGGGGGGCTCCACTCGCATCCCCAAGGTGCAGAAGTTGCTGCAGGACTTCTTCAACGGCAAGGAG
     a q i h d v v l v g g s t r i p k v q k l l q d f f n g k e360
1200 CTGAACAAGAGCATCAACCCTGATGAGGCTGTGGCCTATGGGGCTGCTGTGCAGGCGGCCGTGTTGATGGGGGACAAATGTGAGAAAGTG
     l n k s i n p d e a v a y g a a v q a a v l m g d k c e k v390
1290 CAGGATCTCCTGCTGCTGGATGTGGCTCCCCTGTCTCTGGGGCTGGAGACAGCAGGTGGGGTGATGACCACGCTGATCCAGAGGAACGCC
     q d l l l l d v a p l s l g l e t a g g v m t t l i q r n a420
1380 ACTATCCCCACCAAGCAGACCCAGACTTTCACCACCTACTCGGACAACCAGCCTGGGGTCTTCATCCAGGTGTATGAGGGTGAGAGGGCC
     t i p t k q t q t f t t y s d n q p g v f i q v y e g e r a450
1470 ATGACCAAGGACAACAACCTGCTGGGGCGTTTTGAACTCAGTGGCATCCCTCCTGCCCCACGTGGAGTCCCCCAGATAGAGGTGACCTTT
     m t k d n n l l g r f e l s g i p p a p r g v p q i e v t f480
1560 GACATTGATGCTAATGGCATCCTGAGCGTGACAGCCACTGACAGGAGCACAGGTAAGGCTAACAAGATCACCATCACCAATGACAAGGGC
     d i d a n g i l s v t a t d r s t g k a n k i t i t n d k g510
1650 CGGCTGAGCAAGGAGGAGGTGGAGAGGATGGTTCATGAAGCCGAGCAGTACAAGGCTGAGGATGAGGCCCAGAGGGACAGAGTGGCTGCC
     r l s k e e v e r m v h e a e q y k a e d e a q r d r v a a540
1740 AAAAACTCGCTGGAGGCCCATGTCTTCCATGTGAAAGGTTCTTTGCAAGAGGAAAGCCTTAGGGACAAGATTCCCGAAGAGGACAGGCGC
     k n s l e a h v f h v k g s l q e e s l r d k i p e e d r r570
1830 AAAATGCAAGACAAGTGTCGGGAAGTCCTTGCCTGGCTGGAGCACAACCAGCTGGCAGAGAAGGAGGAGTATGAGCATCAGAAGAGGGAG
     k m q d k c r e v l a w l e h n q l a e k e e y e h q k r e600
1920 CTGGAGCAAATCTGTCGCCCCATCTTCTCCAGGCTCTATGGGGGGCCTGGTGTCCCTGGGGGCAGCAGTTGTGGCACTCAAGCCCGCCAG
     l e q i c r p i f s r l y g g p g v p g g s s c g t q a r q630
2010 GGGGACCCCAGCACCGGCCCCATCATTGAGGAGGTTGATTGAATGGCCCTTCGTGATAAGTCAGCTGTGACTGTCAGGGCTATGCTATGG
     g d p s t g p i i e e v d643.
2100 GCCTTCTAGACTGTCTTCTATGATCCTGCCCTTCAGAGATGAACTTTCCCTCCAAAGCTAGAACTTTCTTCCCAGGATAACTGAAGTCTT
2190 TTGACTTTTTGCGGGGAGGGCGGTTCATCCTCTTCTGCTTCAAATAAAAAGTCATTAATTTATTAAAACTTGTGTGGCACTTTAACATTG
2280 CTTTCACCTATATTTTGTGTACTTTGTTACTTGCATGTATGAATTTTGTTATGTAAAATATAGTTATAGACCTAAATAAGCT 2361
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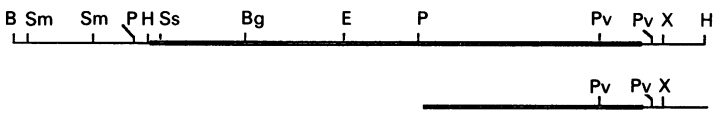
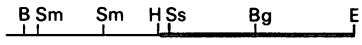
(*b*)

1. HSP70B′ genomic DNA



2. HSP70B′ cDNA

3. HSP70B (Voellmy *et al.*, 1985)



4. HSP70 (Wu *et al.*, 1985)

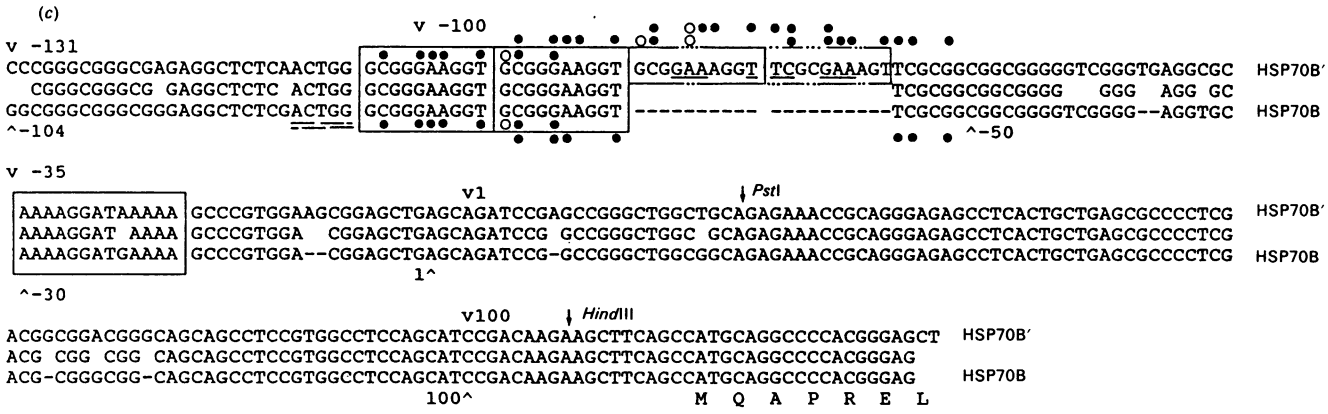


Fig. 2. HSP70B′ DNA sequence analysis

(*a*) Nucleotide sequence of human HSP70B′ gene and predicted amino acid sequence. ▼ refers to the transcription start site (as determined by S1 nuclease mapping; see Fig. 4*a*). The heat-shock consensus sequence starts at nucleotide residue −72 (see Fig. 2*c*; GAA/TTC repeats and the GATA box at position −30) are underlined. HSP70B′ cDNA sequence (1.2 kb) derived from clone WS8 was identical with the genomic sequence from nucleotide residue 1183, marked ↓, to the 3′ end and included a poly(A) tail. (*b*) Restriction maps of HSP70B′ cDNA and gene, the HSP70B gene and the HSP70 gene. Key: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; Sm, *Sma*I; Ss, *Sst*I; X, *Xba*I. (*c*) Comparison of the 5′ upstream sequences of the HSP70B′ and HSP70B genes. Boxed 10-mer repeat and TATA-box-like region are as described by Schiller *et al.* (1988); additional partial repeats in the HSP70B′ gene are indicated by boxes with dotted lines above differing nucleotides. Heat-shock consensus elements are indicated by ●; partial heat-shock-element sequences are also shown, with mismatches indicated by ○. GAA/TTC motifs are underlined. The mRNA start in the HSP70B′ gene was determined by S1 nuclease mapping (Fig. 4*a*). CAAT-like sequence (Schiller *et al.*, 1988) is underlined with double broken lines.

Fig. 3. Comparison of amino acid sequences of human 70 kDa heat-shock proteins

Amino acid residues are numbered according to the human HSP70B′ sequence. Other heat-shock protein sequences were derived from Voellmy *et al.* (1985) (HSP70B; *N*-terminal region only), Hunt & Morimoto (1985) (HSP70) and Dworniczak & Mirault (1987) (HSC70). Amino acid residues that differ from human HSP70B′ are shown, and identical amino acid residues are indicated by a point (.).

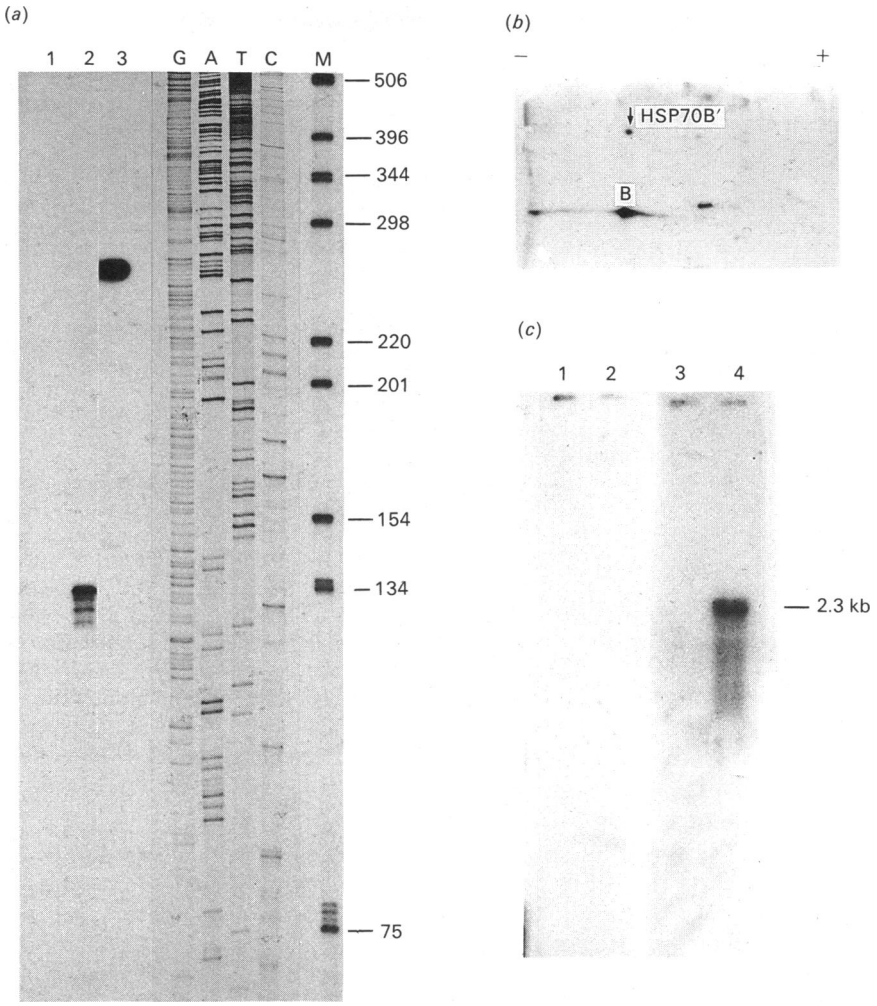


Fig. 4. Analysis of HSP70B′ expression

(*a*) S1 nuclease protection of control and heat-shock mRNA. Portions (50 μg) of control (lane 1) and heat-shock RNA from HeLa cells (lane 2) were hybridized to a 5′-end-labelled 270 bp *Sma*I–*Sst*I HSP70B′ cDNA fragment (lane 3) and treated with S1 nuclease as described in the Materials and methods section. Protected fragments were electrophoresed on a 6% sequencing gel (alongside sequencing and DNA ladders) and autoradiographed for 5–6 h; M, DNA markers sized in nucleotide residues. (*b*) Hybrid selection with specific 3′ HSP70B′ cDNA. mRNA from fibroblasts heat-shocked at 45 °C (see legend to Fig. 1) was hybrid-selected with WS82.3 [600 nucleotide residues extending to the poly(A) tail] and translated *in vitro*, and [^{35}S]methionine-labelled translation products were analysed by two-dimensional gel electrophoresis and fluorography (see Fig. 1). (*c*) Selective hybridization of oligonucleotides to HSP70B′ mRNA. Total RNA (50 μg) from control (lanes 1 and 3) and heat-shocked HeLa cells (lanes 2 and 4) were electrophoresed in formaldehyde/1% agarose gel, transferred to Hybond-N (Amersham International) membrane and hybridized with sequence-specific oligonucleotides complementary to either HSP70B DNA (lanes 1 and 2) or HSP70B′ DNA (lanes 3 and 4) as described in the Materials and methods section.

Expression of HSP70B′ mRNA in human fibroblasts

The expression of heat-shock mRNAs in human fibroblasts in response to stress was investigated by using short 3′ cDNA sequences encoding HSP70, HSC70 and HSP70B′, corresponding to the regions of maximum sequence divergence, in order to differentiate between the various mRNAs in Northern-blot analyses (Fig. 6). The constitutively expressed HSC70 mRNA was increased only very slightly in response to heat-shock at 42 and 45 °C and $CdCl_2$ treatment, with comparable amounts present under all conditions (Fig. 6, lanes 1–4). HSP70 mRNA was induced at 42 °C, and increased to higher levels both after 45 °C and $CdCl_2$ treatment (Fig. 6, lanes 5–8). In contrast, HSP70B′ mRNA, which was strongly induced at 45 °C (Fig. 6, lane 11), was not detectable after 42 °C treatment (Fig. 6, lane 10), and only trace amounts were present after $CdCl_2$ treatment (Fig. 6, lane 12). The possibility of minor cross-reaction between HSP70 mRNA (present in large amounts after $CdCl_2$ treatment) and HSP70B′ 3′ cDNA cannot be excluded in this instance. All three human heat-shock protein 70 mRNAs are of approximately equivalent size (approx. 2.3 kb). The pattern of expression of HSP70B′ and HSP70 mRNAs in heat-shocked fibroblasts (Fig. 6) conformed to the temperature profiles of induction of their transfected promoter constructs expressing reporter chloramphenicol acetyltransferase activity (Fig. 5). HSP70B′-promoted chloramphenicol acetyltransferase activity was strictly heat-inducible; it was barely detectable below 43 °C, but was strongly induced at higher temperatures with maximal amounts at 44–45 °C. In contrast, HSP70-promoted chloramphenicol acetyltransferase activity was not entirely heat-shock-dependent in transfected HeLa cells, but was considerably induced above 42 °C.

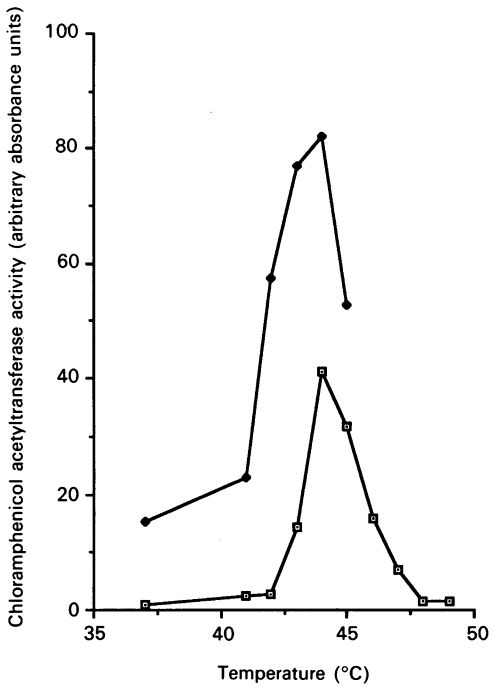


Fig. 5. Promoter activity of pHSP70B′–CAT and pHSP70–CAT in transfected HeLa cells: effect of increasing heat-shock temperature

Chloramphenicol acetyltransferase activity was assayed with [^{14}C]chloramphenicol (0.25 μCi) and acetyl-CoA (4 mM) as substrates on extracts (200 μg) obtained from control and heat-shocked HeLa cells transfected with either the pHSP70–CAT (◆) or the pHSP70B′–CAT vector (⊡). At 40 h after transfection, cells were subjected to heat-shock at various temperatures with 2 h recovery at 37 °C. Radioactive reaction products were extracted, resolved by chromatography on silica-gel t.l.c. plates and autoradiographed. Chloramphenicol acetyltransferase activity was quantified from the radioactive spots of [^{14}C]acetylchloramphenicol on an X-ray film by using a LKB scanner and expressed as arbitrary absorbance units.

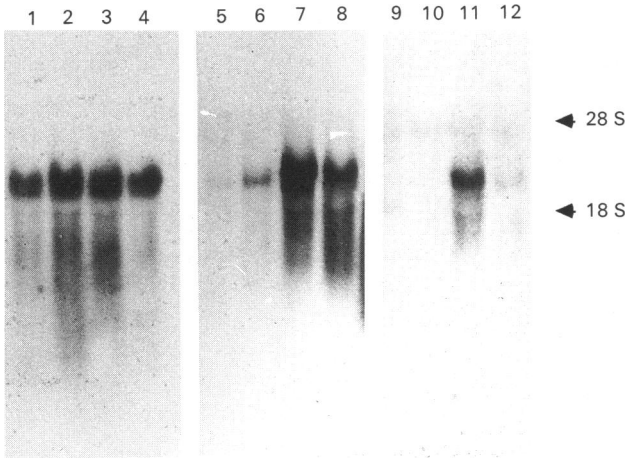


Fig. 6. Northern-blot analysis of RNA from control, heat-shocked and $CdCl_2$-treated fibroblasts

Total cytoplasmic RNA (10 μg) from confluent human fibroblasts (lanes 1, 5 and 9), from cells heat-shocked at 42 °C for 20 min (lanes 2, 6 and 10), from cells heat-shocked at 45 °C for 20 min (lanes 3, 7 and 11) and from cells treated with 100 μM-$CdCl_2$ for 1 h (lanes 4, 8 and 12) was denatured in formaldehyde, electrophoresed in 1% agarose gels, transferred to Hybond-N filter (Amersham) and hybridized to HSC70 cDNA (WS4; lanes 1–4), HSP70 cDNA (WS20; lanes 5–8) and HSP70B′ cDNA (WS8.2.3; lanes 9–12).

DISCUSSION

The novel HSP70B′ cDNA and gene sequence that we have isolated encodes an HSP70 variant that is more basic than HSP70 and has different stress-induction characteristics. There have been several reports of more basic variants of HSP70 (Anderson *et al.*, 1982; Richter & Issinger, 1986; Napolitano *et al.*, 1987), although some of these may be isoforms resulting from post-translational modification such as methylation (Wang *et al.*, 1981). HSP70B′ is clearly the product of a separate mRNA and gene. On the basis of its two-dimensional gel-electrophoresis co-ordinates and expression, which is entirely dependent on heat-shock, HSP70B′ is likely to correspond to HSP72 described by Watowich & Morimoto (1988). These authors also state that HSP72 is the product of a separate mRNA, but no evidence was presented. Our results with respect to (1) the expression of the basic HSP70 variant in human fibroblasts only at 45 °C, (2) the detection of a heat-inducible mRNA by hybridization with a unique 3′ HSP70B′ cDNA fragment as well as with a highly specific oligonucleotide probe, (3) the sequence of the HSP70B′ gene and (4) the promoter activity of the HSP70B′ gene transfected into HeLa cells are consistent with HSP70B′ being a unique member of the heat-shock protein gene family. It is strictly heat-inducible and less sensitive to both $CdCl_2$ and increased temperature than is HSP70. In contrast, the major heat-inducible HSP70 is also expressed constitutively at low levels in HeLa cells and primary lymphocytic cultures (Whatley *et al.*, 1986; Greene *et al.*, 1987; Watowich & Morimoto, 1988) as well as in human post-mortem brain tissue (Whatley *et al.*, 1984).

HSP70B′ has considerable similarity to both the constitutively expressed HSC70 and major heat-inducible HSP70 family members. Its closest homologue, the 5′ genomic fragment described by Voellmy *et al.* (1985), also referred to as HSP70B DNA, has a functional heat-shock promoter that has been well characterized (Schiller *et al.*, 1988; Amin *et al.*, 1988; Brown *et al.*, 1988). However, it is not certain that the latter sequence encodes the same basic heat-shock protein as does HSP70B′ DNA since no cDNA or complete gene sequence has yet been isolated. There are several copies of HSP70 genes in the human genome (Goate *et al.*, 1987; Harrison *et al.*, 1987) and more than one functional gene (Harrison *et al.*, 1987). Our results strongly suggest that HSP70B′ is expressed. A specific heat-induced mRNA homologous to the 5′ end of the Voellmy *et al.* (1985) sequence has been detected by RNAase protection assay (Moore *et al.*, 1987). However, the mRNA detected by this probe could have been transcribed from the closely related HSP70B′ gene. Since both the HSP70B gene (Voellmy *et al.*, 1985) and the HSP70B′ gene have functional heat-shock promoter elements, they may represent two functional genes encoding a second type of heat-shock protein. Nevertheless, only the oligonucleotide probe generated from HSP70B′ DNA, but not that from HSP70B DNA, detected a specific mRNA in heat-shocked HeLa cells.

The promoter regions of the HSP70B′ and HSP70B genes differ considerably from that of the HSP70 gene (Wu *et al.*, 1986) in lacking TATA and CAAT boxes, which are thought to contribute to the basal expression of HSP70 (Greene *et al.*, 1987). Differences between the 5′ regions of the two HSP70B DNA homologues, including several changes localized in key promoter sequences, may generate a subtle regulation in their respective patterns of expression. A G-to-A change at nucleotide residue −27 in HSP70B′ DNA gives rise to a GATA box within the sequence element found in the TATA box region (Schiller *et al.*, 1988). This 13-nucleotide-residue sequence element in the HSP70B gene is thought to induce DNA bending and is part of a DNAase I-hypersensitive region (Schiller *et al.*, 1988; Brown *et al.*,

1988). Point mutations in this sequence appear to affect both structure and transcriptional activity (Schiller *et al.*, 1988). We have evidence obtained by using site-directed mutagenesis of the HSP70B′ gene that mutation of G to T at position −29 to form a TATA box enhances transcription at lower temperatures (M. Y. Rajendran & T. K. C. Leung, unpublished work). A further difference between the two is the 20-nucleotide-residue insertion in the HSP70B′ gene that falls precisely within the heat-shock element of the HSP70B DNA sequence. The partial repeat in this insertion contains a heat-shock consensus sequence and additional copies of the GAA/TTC blocks thought to interact with the heat-shock transcription factor (Sorger & Pelham, 1987; Amin *et al.*, 1988). Overlapping heat-shock-element consensus sequences are a feature of several heat-shock promoter elements (Pelham & Bienz, 1982). This region of the HSP70B′ gene contains three overlapping partial heat-shock-element consensus sequences and one exact consensus. This sequence is likely to promote strong protein interaction, and preliminary investigations indicate that there are differences in stability of protein factor binding between this HSP70B′ DNA sequence and the HSP70 DNA promoter (T. K. C. Leung, unpublished work).

HSP70 family members involved in protein conformational interactions are very similar, particularly in the *N*-terminal region. The divergence of the *C*-terminus of HSP70B′ may contribute to a selective function of this protein, as has been shown for GRP78, where the *C*-terminal four amino acid residues are essential for its retention in the lumen of the endoplasmic reticulum (Munro & Pelham, 1986). The divergent *C*-terminus of HSP70B′, the structural features of the heat-shock promoter region of the gene and its different stress-induction characteristics constitute unique properties of this intriguing addition to the multi-gene heat-shock protein family. The relationship of induction of this gene to the phenomena of stress-induced cellular protein interactions remains to be established.

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