Amplification and altered expression of the *hsc70/U14 snoRNA* gene in a heat resistant Chinese hamster cell line

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Abstract We have recently demonstrated that the heat resistant phenotype of the HR-1 variant isolated from HA-1 Chinese hamster fibroblasts after a series of heat shocks is associated with the increased expression of Hsc70, the constitutive form of Hsp70 (Laszlo and Li 1985). Here, we report the cloning and characterization of the Chinese hamster *hsc70* gene and its organization and expression in wild type HA-1 and permanently heat resistant HR-1 cells. DNA sequencing revealed that the structure and nucleotide sequence of the hamster *hsc70* gene is highly homologous to the human and rat genes coding for Hsc70. Three of the eight introns of the hamster *hsc70* gene encode U14 small nucleolar RNAs, as has been demonstrated in other species. Although putative transcriptional elements, including a TATA box, two inverted CAT boxes, and two sets of heat shock elements (HSEs) are completely conserved in the human and hamster *hsc70* genes, the regulation of expression of the hamster *hsc70* gene is different from that reported for its human counterpart in that the mRNA coding for Hsc70 increases at least 10-fold after a mild heat shock in Chinese hamster cells while no induction of Hsc70 by heat shock has been reported in human cell lines. In situ hybridization revealed a complex chromosomal rearrangement in HR-1 cells which results in the 4- to 5-fold amplification of the *hsc70* gene as indicated by genomic Southern blots. In association with this amplification of the *hsc70* gene, the levels of Hsc70 mRNA and U14 snoRNA are increased in the HR-1 cells under both normal growing conditions and after heat shock. Thus, the elevated expression of both Hsc70 and U14 snoRNA might play a role in the heat resistant phenotype of the HR-1 cells. This is the first report of the amplification of a heat shock gene and the possible induction of gene amplification by heat shock.

INTRODUCTION

In all organisms examined so far, an increase in the expression of a small set of proteins, called the heat shock proteins (Hsps), occurs in response to heat shock or certain other environmental stresses, which include heavy metals, oxidizing agents, amino acid analogs, viral infection and certain organic solvents (Linguist and Craig 1988; Morimoto et al 1990, 1991; Nover 1991). The family of proteins whose molecular weights are close to 70 kDa, the so-called Hsp70 family, represent a group of

abundant cellular Hsps. The members of this family have been implicated in the processes of protein translocation, protein-protein interactions, protein folding, and repair of stress-induced damage in proteins, although it is not yet clear to what extent the stress inducible or constitutive forms of Hsp70 play this role (Brown et al 1993; Morimoto 1993; Pelham 1984). In mammalian cells, the expression of the various members of this multi-gene family appears to be subject to various regulatory mechanisms. The expression of the heat inducible Hsp70 is usually very low, or undetectable under normal growth conditions, and it is induced rapidly and greatly upon heat shock in most mammalian cells (Hunt and Morimoto 1985; Hunt and Calderwood 1990; Morimoto 1993). However, in the case of primates (including humans), there is a basal level of the expression of Hsp70, which appears to be increased

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in transformed cells and is thought to be due to the presence of a serum regulated element in the promoter region of the gene (Wu and Morimoto 1985). In contrast, the so-called cognate form of Hsp70, Hsc70, is constitutively expressed under normal growth conditions and its expression has been reported to be only slightly increased after heat shock in human, mouse and rat cells (Dworniczak and Mirault 1987; Sorger and Pelham 1987; Giebel et al 1988). Expression of the members of the Hsp70 family can also be tissue specific, as is the case with the *hsp70t* gene which is expressed in testes and ovaries (Zakeri and Wolgemuth 1987; Zakeri et al 1988; Wiesniewski et al 1990; Rosario et al 1992).

An important feature of the genes coding for Hsc70 in vertebrates is that they contain introns, unlike the genes coding for the other members of the Hsp70 family (Dworniczak and Mirault 1987; Sorger and Pelham 1987; Giebel et al 1988; Zafarullah et al 1992). Introns 5, 6 and 8 contain highly conserved repeats of about 90 bp which have been recently identified to encode the U14 snoRNA in the mouse *hsc70* gene (Liu and Maxwell 1990). U14 snoRNA is a low molecular weight RNA species of 80–150 nucleotides capable of intermolecular base-pairing with mRNA and 18 S rRNA (Liu and Maxwell 1990; Leverette et al 1992). Since the expression of U14 snoRNA appears to be regulated by the same transcriptional elements as Hsc70 mRNA in mammalian cells (Leverette et al 1992), we shall call this the *hsc70/U14 snoRNA* (abbreviated as *hsc70/U14*) gene in this report.

Our laboratory has been interested in the role of various members of the Hsp70 family in transient thermotolerance and permanent heat resistance (Laszlo and Li 1985; Laszlo 1988a, 1988b). Thermotolerance, the acquisition of a transient resistance to a subsequent lethal heat challenge after an exposure to a non-lethal heat shock, has been found in both prokaryotic and eukaryotic cells (Morimoto et al 1990). Although the exact mechanisms involved in thermotolerance have not been established, several lines of evidence have suggested that the Hsp probably play a role in both transient thermotolerance and permanent heat resistance. For example, cell lines expressing elevated levels of Hsp27, Hsc70, and Hsp90 have been found to be heat resistant (Laszlo and Li 1985; Yahara et al 1986; Chretien and Landry 1988) and rodent cells transfected with either human Hsp70 or human Hsp27 are permanently heat resistant (Landry et al 1989; Li et al 1991, 1992). Among the Hsps, the level of Hsp70 expression correlates best with thermotolerance (Laszlo and Li 1985; Li 1985). However, it is not clear whether or not all, or only certain, members of the Hsp70 family play a role in thermotolerance. Hsc70 under normal growth conditions is expressed at a higher level in heat resistant HR-1 Chinese hamster cells (initially called heat resistant variant 3012) (Laszlo and Li 1985). The same gene product

is constitutively expressed and induced by heat shock and other stresses in cell lines derived from Chinese hamster ovaries (Laszlo and Li 1985; Laszlo 1988a; Fornace et al 1989; Ahmad et al 1990). The inducibility of the *hsc70* gene by heat shock in Chinese hamster cells contrasts with findings in human, mouse, rat and fish cells, in which no significant induction of Hsc70 by heat shock has been observed (Dworniczak and Mirault 1987; Sorger and Pelham 1987; Giebel et al 1988; Zafarullah et al 1992). This unusual pattern of Hsc70 expression in hamster cells could result from alteration(s) in the structural gene coding for Hsc70 and/or from alterations in its regulatory mechanisms. Furthermore, the genetic basis of the heat resistant phenotype associated with the over-expression of Hsc70 in the HR-1 hamster cells is yet to be established. In an initial approach toward answering these questions, we have cloned and characterized the *hsc70/U14* gene from Chinese hamster cells and characterized its chromosomal organization in both wild type (HA-1) and heat resistant variant (HR-1) cells. Furthermore, the expression of Hsc70 and U14 snoRNA in HA-1 and HR-1 cells under a variety of experimental conditions was also examined.

MATERIALS AND METHODS

Cell culture and heating

The Chinese hamster cell lines CHO-K1, HA-1 and HR-1 were cultured as previously described (Laszlo and Li, 1985). Essentially, HA-1 and HR-1 cells were grown in Dulbecco's modified Eagle's Medium, supplemented with 10% fetal calf serum, non-essential amino acids and gentamycin (5 µg/ml). CHO-K1 cells were grown in Ham's F-12 medium, 10% fetal calf serum and gentamycin (5 µg/ml). Exponentially growing cells were used throughout these experiments. The medium of the culture was changed just before heating using pre-warmed (37°C) medium; cell monolayers were then incubated for 10 min in a 37°C water bath. The cells were then heated at the appropriate temperature (usually 45°C) by immersion into a precisely controlled water bath ($\pm 0.08^\circ\text{C}$) for appropriate lengths of time. Following heating, the monolayers were incubated in the 37°C water bath for 10 min, followed by another medium change using the pre-warmed medium and allowed to recover for various lengths of time at 37°C.

DNA library screening and DNA sequencing

About 10^6 primary clones from a CHO genomic library made in the vector λ fix (obtained from Stratagene) and about 10^5 primary clones from another CHO genomic library (made in the vector λ charon 4, a gift from Dr P. Wejksnora, University of Wisconsin-Milwaukee,

Milwaukee, WI) (Wejksnora 1985) were screened with a probe made by random primer labeling a full length hamster *hsc70* cDNA (a gift from Dr R. Gupta, McMaster University, Hamilton, Ontario) (Ahmad et al 1990) following standard methods (Ausubel et al 1987). Fifty-four positive clones were obtained from screening these two libraries. Recombinant phage DNAs were isolated from small-scale liquid lysates and mapped with *EcoR* I and *Hind* III (Promega) by Southern blots using either the 5' or 3' ends of the *hsc70* cDNA as probes. Four representative clones were selected for further analysis. Their inserts were partially digested with *Sau*3 A and subcloned into the *Bam* HI site of Bluescript KS phagemid (Stratagene). Positive subclones were selected by colony hybridization using appropriate probes and sequenced with Sequenase version 2.0 (USB) using T3 and T7 universal primers and other specific synthetic primers.

Sequence analyses such as multiple alignments and similarity searching were conducted using programs of the DNASTAR package version 2.0 (DNASTAR Inc, Madison, WI).

Genomic DNA isolation and Southern blot analysis

High molecular weight DNA from CHO, HA-1, and HR-1 cells was isolated by the salting out method (Miller et al 1988) and digested with various restriction enzymes. The digested DNA samples were then separated on a 0.7% agarose gel in TBE buffer and transferred to a Gene Screen Plus membrane (NEN). The blot was hybridized with the full length *hsc70* cDNA probe generated as described above, in 10% dextran sulfate/1%SDS/1M NaCl at 65°C. The filter was washed with 0.1 × SSC/1%SDS solution at 65°C for 2 h and exposed to Kodak SR-5 X-ray film for 48 h. In some experiments, the filter was stripped and then rehybridized with a probe made by random primer labeling from the first intron and the 5' upstream region of the *hsc70* gene contained in clone 19-2a (from residue 1 to residue 1129, Fig. 4).

RNA purification and Northern blot analysis

Cell monolayers were lysed by the addition of 5 M guanidine thiocyanate to the dishes and total cellular RNA was isolated as described previously (Laszlo 1988a). Equal amounts (5 µg) of total RNA from control and heated cells were separated on a 1.5% agarose gel containing 1% formaldehyde, 40 mM 3-[N-morpholino] propane sulfonic acid (MOPS), 10 mM sodium acetate, 1 mM EDTA, pH 7.0, blotted onto nitrocellulose, and then hybridized with a probe made by random primer labeling of a section of the 3'-non-coding region of the *hsc70* gene (from residue 4340 to residue 4553, Fig. 4). Hybridization and washing conditions were identical to those described above for

the Southern blots. The filter was stripped and rehybridized with a probe made by the same method from the insert of clone pM3, a 5' truncated *hsp70* cDNA clone (Fornace et al 1989) under similar conditions. The nucleotide sequence of the insert of pM3 indicated that it had 85% and 65% identity to the mouse *hsp70* gene (Hunt and Calderwood 1990) and the mouse *hsc70* cDNA (Giebe et al 1988), respectively (M.-S. Chen and A. Laszlo, unpublished observations). The filter was also probed with a β -actin cDNA (a gift from Dr K. O'Maley, Washington University, St. Louis, MO) to monitor the relative loading of the different RNA samples.

For analysis of U14 snoRNA, equal amounts of total cellular RNA were separated on a 10% polyacrylamide gel in TBE, electroblotted onto a Gene Screen Plus (NEN) membrane and hybridized with a U14 snoRNA probe generated by the polymerase chain reaction using a pair of primers which cover the region from residue 2749 to residue 2826 of the hamster *hsc70/U14* gene (Fig. 4).

Fluorescent in situ hybridization

Labeling of DNA probes and fluorescent in situ hybridization (FISH) were performed as described (Jauch et al 1992; Featherstone and Huxley 1993). Briefly, metaphase spreads were made from HA-1 and HR-1 cells by standard methods and stored in 70% ethanol at 4°C until use. DNA from clone 19-2a representing the entire hamster *hsc70/U14* RNA gene in a 20 kb insert (see below) was labeled by incorporation of biotin-11-dUTP (Sigma) using a nick translation kit (GIBCO-BRL). The DNase concentration in the nick translation reactions was established empirically to yield probe fragments in the size range 100–600 bp, as determined by agarose gel electrophoresis under denaturing conditions.

Before hybridization, slides were treated with RNase A (Boehringer-Mannheim) (100 µg/ml in 2 × SSC), followed by pepsin (50 µg/ml in 0.01 M HCl) at 37°C for 1 h and 10 min respectively, washed in PBS, fixed in 1% formaldehyde and dehydrated through an ethanol series. Hybridizations were performed with 100 ng of biotin labeled DNA, plus 30–60 µg hamster genomic DNA, 5 µg of sheared salmon sperm DNA and 5 µg of *Escherichia coli* tRNA (both from Sigma), in 10 µl of 10% dextran sulfate, 50% formamide in 2 × SSC. This mixture was denatured at 73°C for 5 min, pre-annealed at 37°C for 20 min and applied to metaphase spreads which had been denatured by a 2-min treatment in 70% formamide in 2 × SSC at 70°C followed by ethanol dehydration. Hybridization was allowed to proceed overnight at 37°C, followed by three 5-min washes in 50% formamide in 2 × SSC at 45°C, and three 5-min washes in 0.1 × SSC at 60°C. Slides were blocked with 5% BSA/4 × SSC for 30 min at 37°C and incubated with 10 µg/ml of fluorescein isothi-

ocyanate (FITC) conjugated avidin (Vector Laboratories) in $4 \times \text{SSC}$ containing 0.2% Tween-20 for 30 min at room temperature, followed by three washes in $4 \times \text{SSC}/0.2\%$ Tween-20 at 45°C for 5 min. The biotin signal was amplified using a sandwich technique (Pinkel et al 1986). Finally, slides were stained in DAPI ($10 \mu\text{g}/\text{ml}$) and mounted in antifade for observation.

Slides were examined using a Zeiss Axiovert fluorescent microscope. The DAPI banding was viewed with a '02' filter set, the FITC signal with a '09' filter set. Images were captured using a cooled CCD (Photometrics, Tucson, AZ) and processed as described previously (Featherstone and Huxley 1993).

Nucleotide sequence accession number

The nucleotide sequence reported in this paper is entered in the GenBank nucleotide sequence base with accession number L19533.

RESULTS

Isolation and characterization of Chinese hamster *hsc70/U14* gene

Of the 54 positive recombinant clones which were identified from the screening of two different genomic DNA libraries using the full length *hsc70* cDNA as a probe, four representative clones which hybridized to both 5' and 3' end-specific probes of the hamster *hsc70* cDNA were selected for further analysis. This included restriction mapping, subcloning, and sequencing. One of these clones, clone 19-2a, was found to contain the entire *hsc70/U14* gene. Partial sequencing of the other three clones revealed that they contain intron-less DNA fragments which are 90–95% homologous to the hamster *hsc70* cDNA (data not shown) (Ahmad et al 1990). Two of these clones were found to contain at least one internal stop codon at the 5' end of the coding region, while the other clone contained a deletion which caused a reading frame shift within the 5' coding region that was sequenced (data not shown). These results strongly suggest that these three clones contain *hsc70* pseudogenes.

The detailed restriction map, structural organization, and the entire nucleotide sequence of the Chinese hamster *hsc70/U14* gene contained in clone 19-2a are shown in Figures 1 and 4, respectively. The structural organization of the Chinese hamster *hsc70/U14* gene is very similar to that found in rat and human cells: the eight introns are located in exactly the same positions relative to the protein coding regions as found in the rat and human *hsc70/U14* genes (Dworniczak and Mirault 1987; Sorger and Pelham 1987).

Detailed sequence comparison of both the nucleotide sequences and the putative amino acid sequences for

Fig. 1 Restriction map and structural organization of the Chinese hamster *hsc70/U14* gene contained in clone 19-2a. Restriction enzyme sites are indicated as follows: B = Bam H 1, E = Eco R 1, P = Pst 1, and S = Sma 1. The boxed regions represent exons with the coding region in black. The eight introns of this gene are indicated by I1 to I8. U1, U2 and U3 represent the three intron regions which encode U14 snoRNAs.

which they code, further confirmed that the gene in clone 19-2a is the Chinese hamster counterpart of the rat and human *hsc70/U14* genes. The amino acid sequence of the protein encoded by this gene is identical to that encoded by the human *hsc70/U14* gene, except for a change at residue 111, which is an alanine in the Chinese hamster Hsc70 instead of a threonine in the human Hsc70 (Dworniczak and Mirault 1987). The 5' upstream region of the hamster *hsc70/U14* gene contains several putative transcriptional control elements, including a TATA box, two inverted CAT boxes, and two sets of heat shock elements (HSEs) (Figs 4 and 5). The nucleotide sequences of these putative transcriptional regulatory elements are completely conserved when compared to the analogous regions of the rat and human *hsc70/U14* genes (Fig. 5). (11,59). Alignment of the nucleotide sequences of introns 5, 6, and 8 (Fig. 4, underlined region) revealed that the hamster U14 snoRNA coding sequences have over 60% identity with their counterparts in mice, rats and humans (data not shown).

There is a difference in eight nucleotide residues between the nucleotide sequences of the exons of the *hsc70/U14* gene contained in clone 19-2a and the previously reported nucleotide sequence of *hsc70* cDNA of Chinese hamster cells (Ahmad et al 1990). Five of these differences are located within the coding region. Of these five base pairs, three represent neutral mutations and two represent a GC inversion resulting in a change of two residues in the amino acid sequence (residues 745 and 746 of the *hsc70* cDNA as shown in Figure 5A of Ahmad et al 1990 and residues 2300 and 2301 in the genomic DNA as shown in Fig. 4 of this report). The position of these two residues in sequencing reactions performed from the anti-sense direction was reversed because of gel compression. Thus, this GC inversion might be an artifact in the cDNA sequence. Moreover, detailed examination of this region using two synthetic

Fig. 2 Metaphase chromosomes of HA-1 (A) and HR-1 (B) cells challenged with the biotin-labeled genomic insert of clone 19-2a. Arrowheads point to the location of *hsc70/U14* gene in HA-1 (A) and HR-1 (B) cells; the arrow indicates the site of amplification of *hsc70/U14* gene which may also be the break point at the original locus involved in the generation of a new large metacentric chromosome (open arrowhead) (see text).

primers clearly determined that the nucleotide sequence was GC instead of CG (data not shown). The other neutral mutations might be due to different alleles or some other polymorphism associated with the different CHO sub-lines which were used to generate the respective libraries.

Amplification of *hsc70/U14* gene in HR-1 cells

Equal amounts of genomic DNA extracted from three different Chinese hamster ovary-derived cell lines, CHO, HA-1 and HR-1, were digested with various restriction enzymes and subjected to Southern blot analysis with various *hsc70*-related probes (Fig. 6). When the full length *hsc70* cDNA was used as a probe, several hybridizing bands were observed. This result indicated the presence of a family of Hsc70-related sequences in these three cell lines (Fig. 6A) and also confirmed the presence of the pseudogenes identified above. However, when the same filter was stripped and rehybridized with a probe made

Fig. 3 Hybridization of interphase nuclei with biotin-labeled genomic insert of clone 19-2a. The arrowheads indicate the location of the signal from the normal *hsc70/U14* gene in HA-1 (A) and HR-1 (B) interphase cells, while the arrow in (B) indicates the location of the signal from the amplified *hsc70/U14* gene in HR-1 interphase cells.

Fig. 4 Nucleotide sequence of the *hsc70/U14* gene and the deduced amino acid sequence of the protein. The exon/intron boundaries were determined by comparison with the cDNA sequence (Ahmad et al 1992). Putative transcriptional regulatory elements and poly(A) addition sequences are underlined and represented as follows: A, inverted CAT boxes; B, heat shock elements (HSE); C, Sp1 elements; D, TATA box; E, poly(A) addition sequences. The sequences which encode the U14 snoRNAs within introns 5, 6, and 8 of this gene are bold and underlined.

CCCGAACGCGGAGAACACTGCTCTCATTTGGTCCGCTGTGAATCCACCGCCTCCCTTCGT	60	GAGATGCTAAACTAGACAAGTCACAGATCCATGACATTGTCTTGGTGGGTGGTTCCACCA	2580
CGGCTTCCTTCGCTCCCTCCCGCAGCGTGACTGGGCGCGCATGCTACAGGCTGCCG	120	D A K L D K S Q I H D I V L V G G S T R	341
AGTCCCTCCCTCCCGCGCAGCGTGTGAGTGACAGAGGACTCCCGCGCGGGCTCTCGG	180	GAATCCCCAAGATTCAGAAGCTTCTGCAAGACTTCTTCAATGGAAAAGAACTGAATAAGA	2640
CGCCCGCCCTCCCTTTCCCGCGGCGTTCGGAGAGCGGGGCGAGCATGAAAGTTCC	240	I P K I Q K L L Q D F F N G K E L N K S	361
AGAACCGTCGGACGTGCGTCATCGCCAGGTGGGGCGGGCGAGGGCGGACCTAGGAGA	300	GCATTAAACCCCGATGAAGCTGTGCTTATGGTGCGAagtaaccactacatagttgtctt	2700
GCGTCTCGGGGTTCTATTGGTGTGTCGAAAAGCCCATCGTCCATTGGCTAAGGAGCC	360	I N P D E A V A Y G A A	373
ATGATAGGCAGCGGGGCGGGGCTCTTCTGGAAGGTTCTGAGACAGGGTATAAGAG	420	tacagtctagcaagtcctctttaaagagtgctctaaatcgctatgatgatggattcca	2760
ACAGTAGGCGGGCGGAAACCGGTCTCATTTGAACGCGGAGGAGCTTGTGAGGTTTGTG	480	aaaccattcgtagtttccaccagaatgctgtgttggttagtctccttcttgatgctctg	2820
TGGTCTGTCGCCAGCAGCGGGGCTACAGGCAAGtgaggcctggggttgggtgtg	540	agcgaactgaactgcattgaagtgtggttggaactgggtggtgagtttctaacatttctt	2880
ggcgggtggcctgcttactgcagcagagcggggactgagcgggcgggggtggt	600	ccacttttacagCTGTCCAGGCGGCCATTCTATCTGGAGACAAGTCTGAGAATGTTACGG	2940
taggcgcggcctcggggacccggagctgcagctgaggaggaagggttaggtaggcg	660	V Q A A I L S G D K S E N V Q D	389
gcgcgcgaatggcctgagcctgctgcggcgaggaggggtgcccgcgcgcgcgcgcgc	720	ATTTGTGCTCTTGGATGTCACTCTCTTCCCTTGGCATGAAACTGCTGGTGGAGTCA	3000
gctccctttcacccgctcgcggattaaacgagaggggttctgaccgcgcccaaggcgga	780	L L L L D V T P L L S L G I E T A G G V M	409
gctccttggaattattcagcaggcgagctgcggcgccgaatgcgcggtcctggcgctgg	840	TGACTGTCTCATCAAGCGCAATACCACCATCCCTACCAAGCAGACGAGACTTCACTA	3060
aaggggggaggggttgggttctccaggttgaggccacgtgctcaaccattgtgcagacc	900	T V L I K R N T T I P T K Q T Q T F T T	429
gtcaggacagatggttctcagccagggcggggtctgcagggttggcagcggtaccgg	960	CCTATTCTGACAATCAGCGCTGGTGTGCTCATTACgatttttagaattttgtttgatttt	3120
actgcagcagtgccgcctgagcctttgctggcctcaggtgataggtctgggtgagt	1020	Y S D N Q P G V L I Q	441
agtcacatcggaattcggggtctgggaatgagctgctagccgttttcggtgtt	1080	ctagaaatcccggaatgaagtcctgctgtagatgagttcttaaccattcgtagtt	3180
ggttttctttcagCAACCATGTCTAAGGACCTGCAGTTGGTATTGATCTTGGACCAACC	1140	tcaccagaatctctgttggctagttccttcttgatgctcagcagagaattctttc	3240
M S K G P A V G I D L G T T	14	ccctaattgagcttaattctacagaactgtttttctaagctgatgacttatttctgtg	3300
TACTCTGTGTGGGTGTCTCCAGCATGGAAGGTGGAGATAATTGCCAATGACAGGGT	1200	ttccttcaagTCTATGAAGGCGAAAGGGCCATGACCAAGGATAACAACCTGCTTGGAAA	3360
Y S C V G V F Q H G K V E I I A N D Q G	34	V Y E G E R A M T K D N N L L G K	458
AACCGAACCCGCAAGCTATGTGCTTTTACTGACACAGAACGATTAATGGGGATGCC	1260	GTTTGAACCTACAGGCATCCCTCCAGCACCCCGGGTGTCTCAGATTGAAGTTACTTT	3420
N R T T P S Y V A F T D T E R L I G D A	54	F E L T G I P P A P R G V P Q I E V T F	478
GCGAAGATCAGGTGCAATGAACCCACCAACACAGTTTTTGTgaagtagccagctttt	1320	TGACATTGATGCCAATGGCATCCTCAATGTCTCTGCTGTAGATAAGAGCACAGGCAAGGA	3480
A K N Q V A M N P T N T V F D	68	D I D A N G I L N V S A V D K S T G K E	498
acgtgggtagaagctctctagattactgtagtttaagagaagtaacgtactgataaaa	1380	GAACAAGTCACTATCACCACGACAAGGgaagtagtgagcataactcctgctgatgtg	3540
cccgggcagttatttttggtattaaatcgggccaactccttctgacccggagcttagcc	1440	N K I T I T N D K G	507
tcgcttagccgtagtgcgcttttcttccggccctggttttggtagggaccaactccc	1500	gcttctgcttacaagtagttattgacttttagtttagtttagtttagtttagtttagttt	3600
tttagtgtacaatttacttcacaactctcagcctcggaatttaattggtggagtttaata	1560	gtgacttaacagCGCGCTTGAGCAAGGAGGATATTGAACGCATGGTCCAGGAAGCTGAGA	3660
gatacacacttattttgcagATGCCAACGCTGTGATCGGACGTAGGTTTGTGATGCTGT	1620	R L S K E D I E R M V Q E A E K	523
A K R L I G R R F D D A V	82	AGTACAAAGCTGAAGATGAGAAGCAGAGAGATAAGGTTTCTCCTCAAGAATTCATGGAGT	3720
TGTTCACTGTATATGAAGCACTGGCCCTTCACTGGTGAACGATGACGAGGAGGCTCTAT	1680	Y K A E D E K Q R D K V S S K N S L E S	543
V Q S D M K H W P F M V V N D A G R P K	102	CCTATGCTTTCAACATGAAGCAACAGTTGAAGACGAGAACTTCAAGGCAAGATCAATG	3780
GGTCAAGTAGAATACAAAGGAGGCAAAAGTTTCTACCCAGAGGAGGTGCTCTAT	1740	Y A F N M K A T V E D E K L Q G K I N D	563
V Q V E Y K G E A K S F Y P E E V S S M	122	ATGAGACAACACAGAGATTCTAGACAAGTGAATGAATCACTAGTTGGCTGGATAAGA	3840
GGTTCTGACCAAGATGAAGGAAATTGCAAGAGCTATCTCGGGAAGgtgggttttaactg	1800	E D K Q K I L D K C N E I I S W L D K N	583
V L T K M K E I A E A Y L G K	137	ATCAGgtaggtgctcgtgttgccaataaggggaaggtagtagactagactgggctaaagta	3900
ctgtcaatattgggtggtctattcaagcattcttctgttcagcgccaatttctgaaggta	1860	Q	585
acaattgtgtgattgtattttctagACTGTATTCAATGCTGTGGTCACAGTCCAGCCTA	1920	taggggtcactgtgtagaatggtccaaacattctcggtttccaccagaatgtaaaagcagt	3960
T V T N A V V T V P A Y	149	gttggaattaccttcttggatgtctgagtagcttaagtagtaacggaagtaggttctt	4020
CTTCAATGATTCTCAGCGCAGGCTACAAAGATGCTGGAACATTGTGCGCTGAATGT	1980	gttagttacataggcttaatttgcagccttcagaagtttaactgtttgtggaccatttac	4080
F N D S Q R Q A T K D A G T I A G L N V	169	gtaatgttttttttctactcagACTGCAGAGAAGGAAGTAATTGAACACCAGCAGAAA	4140
CCTGCGAATTATCAATGAACCAACTGCTGCTGCTATTGCTTATGGGCTAGATAAAGAGT	2040	T A E K E E F E H Q K	597
L R I I N E P T A A A I A Y G L D K K	188	GAACGGAGAAGGTCTGCAACCCCATCATTACCAAGCTGTACCAGAGTGTGGTGGCATG	4200
atgtgttttcaagcacatgttttgccttaagattaatttgcacaccagagatgtgtagctaaa	2100	E L E K V C N P I I T K L Y Q S A G G M	617
agtttttctttcattgacagGTTGGAGCTGAAGGAATGTGCTCATTTTGTACTTGGAG	2160	CCTGGAGGAATGCCGGGGGCTTCCCTGGTGGTGGAGCTCCTCCATCTGGTGGTCTTCT	4260
V G A E R N V L I F D L G G	201	P G G M P G G F P G G G A P P S G G A S	637
GTTGACTTTTGTGCTCTCTCTACTATGAGGATGGAATTTTGAAGTCAAAATCAA	2220	TCAGGCGCTACCATTGAAGAGGTGGATTAAGTCAGTCCAAGTAGAGGTGTAGCATGTTTC	4320
G T F D V S I L T I E D G I F E V K S T	221	S G P T I E E V D TER	646
CAGCTGGAGACACCCACTTGGGTGGAGAGACTTTGATAACAGGATGGTCAACCAATTCA	2280	CACAGGGAAACATTGAAGGACCCAAATTTGCAAGTTTCATGGCAGTTTTTAATTTAA	4380
A G D T H L G G E D F D N R M V N H F I	241	ACTGTTAAACAATAATTACTGGGCTTCTCACTACTGAACATGGAACATGTACACAGGA	4440
TTGCTGAGTTTAAGCGAAAGCACAGAAGGACATCAGCGAGAACAAGAGAGCTGTACGGC	2340	AATAAAACATTGCACCTTATAAGCACTGTATCCTAAGTGGAAAACGCAATGTCTAATATG	4500
A E F K R K H K K D I S E N K R A V R R	261	GTGGAAAATGGAATGTCTAAATAAACTATTTAATTTGGCACCATACAGTTGTCTGACT	4560
GTCTGCTACTGCTGTGAACGGGCAAGCGCACCTCTCTCCAGCACCCAGGCCAGTA	2400	A A C T T T G G A A T T A A G G T G G C T T T T G T C A A G G A C A A A T T A C A A A T G C A G G A A T G A G A G	4620
L R T A C E R A K R T L S S S T Q A S I	281	ATACTACCTTAACACAATTCTAGTACCTCCAGTGGGTTTTTCAGCTTAGCCTTAGGCTA	4680
TTGAGATTGATTCTCTATGAGGGAATTGATTCTATACCTCCATTACCCGTGCTCGAT	2460	AGAACCAACATGAGTTCAAAGCTGAAGGTGCTGGG	4715
E I D S L Y E G I D F Y T S I T R A R F	301		
TCGAAGAAATGAATGTGACCTGTTCCGAGGCACTCTGGACCTGTAGAGAAGGCCCTTC	2520		
E E L N A D L F R G T L D P V E K A L R	321		

Rat		<u>CATTGGTCCGTTGCGG</u>	-401
Hamster		<u>CATTGGTCCGCTGTGA</u> A	-402
Rat	GTGTACCCGCC-CTCCTTCATGGGCATCCTTCCGCTCCCTCCCGCCTGCGCTGACGGGTC		-342
Hamster	ATCCACCCGCCTCTCCTTCGTCGGCTTCCTTCCGCTCCCTCCCGCACGCGCTGACTGGGC		-342
Rat	ACGCATGCGTACTCGGTGCCGACGCTCTCCCTTCCCCACCAGCTCTGAGTGACAGAGGA		-282
Hamster	GCGCATGCGTACAGGCTGCCGAGTCCCTCCCCTCCCCCGCCAGC-GTGAGTGACAGAGGA		-283
Rat	CTCCCGCGCGCGGGCTCTCGGCGCCCCGCCCTCCCCTTTCCCCGGGCGGTTCCGAGAGC		-222
Hamster	CTCCCGCGCGCGGGCTCTCGGCGCCCCGCCCTCCCCTTTCCCCGGGCGGTTCCGAGAGC		-223
Rat	GGGGGCGAGCGTGAAAGTTCCAGAACGCTGCGGTC-GTGCGTCATCGCCCGGAGG---GG		-166
Hamster	GGGGGCGAGCATGAAAGTTCCAGAACGCTGCGGTC-GTGCGTCATCGCCAGGTGG---GG		-167
Human	GAGCTTGAAAGTTCCAGAACGCTGCGGTGAGTGCGTTATCGTGAGGCGGCGCGG		-175
		B	
Rat	CGGAGTGAGGCGGGTCC----TAGGAGAGC-GT-TCTGG---TTGTTCATTA-G--TGC		-118
Hamster	CGGGGCGAGGCGGGTCC----TAGGAGAGC-GTCTCGGGGCG--TTCATTG-G--TGC		-118
Human	TGGGGTGGGTGCGGAAGGGGCGAGGCGAGGAGTGAGCCGCGTTGT-GATTGTGATTGG		-114
Rat	A-CTCCGGAGAACCTGGCCG-TCCATTGGCTAAGGAGCTC-TGATCGGCAGCGGGATGGG		-61
Hamster	-TGTTGCAAAAGCCCCATCG-TCCATTGGCTAAGGAGCCA-TGATAGGCAGCGGGACGGG		-61
Human	GTCTTGTAAGGGCAGCCGACTCTATTGGCCGGGAACCTAATG-CAGGAAGCA-----G		-61
		A	
Rat	GCGGAACCCCTTCTGGAAGGTTCTAAGACAGGGTATAAGAGACAGTGAAGCGGGCGGAAAC		-1
Hamster	GCGGGATCCTTCTGGAAGGTTCTAGACAGGGTATAAGAGACAGTGAGGCGGGCGGAAAC		-1
Human	GCGGACCCCTTCTGGAAGGTTCTAAGATAGGGTATAAGAGGAGGGTGGCGGGCGGAAAC		-1
		B D	

Fig. 5 Nucleotide alignment of the 5' upstream sequences of the Chinese hamster, rat and human *hsc70/U14* genes. The symbols :, * and + represent identical, unmatched, and transitional residues, respectively. Putative transcriptional regulatory elements are underlined and represented as follows: A, inverted CAT boxes; B, heat shock elements (HSE); D, TATA boxes.

from the first intron and the 5' non-coding region of the *hsc70* gene contained in clone 19-2a (from residue 1 to residue 1129, Fig. 4), only one hybridizing band was detected, except in the sample digested with Bam H1, which had two hybridizing bands (Fig. 6B, lanes 4, 8 and 12). These two bands reflect the presence of an internal Bam H1 site (at residue 384, Fig. 4) within the region covered by the probe used in this experiment. These results indicate that the *hsc70/U14* gene found in clone 19-2a is present as a single copy in the Chinese hamster genome.

While the overall hybridization patterns obtained when the entire *hsc70* cDNA was used as a probe are very similar to each other in the three cell lines tested, one or two bands in the DNA isolated from HR-1 cells (depending on the restriction enzyme used) appear to hybridize more intensely than in the DNA obtained from HA-1 and CHO cells (compare lanes 9-12 with lanes 1-4 and lanes 5-8 in Fig. 6A). These bands, displaying increased hybridization intensity in the DNA isolated from the HR-1 cells, represent the *hsc70/U14* gene, because they specifically hybridize with a probe made from the 5' non-coding

Fig. 6 Southern blot analysis of *hsc70/U14* gene-related sequences in various Chinese hamster cell lines. Ten μ g of high molecular weight DNA isolated from CHO-K1 cells (lanes 1–4), HA-1 cells (lanes 5–8) and HR-1 cells (lanes 9–12) was digested with Eco R1 (lanes 1, 5, and 9); Pst 1 (lanes 2, 6, and 10); Hind III (lanes 3, 7, and 11); and Bam H1 (lanes 4, 8, and 12). The digested DNA samples were separated on 0.7% agarose gel and transferred to Gene Screen Plus membrane. Lane 13 contained DNA size markers. In panel A, the blot was probed with the full length cDNA of Chinese hamster *hsc70*. In panel B, the same filter was stripped and re-hybridized with a probe made from the first intron and the 5' upstream region (from residue 1 to residue 1129, Fig. 4) of the *hsc70/U14* gene.

region of clone 19–2a (from residue 1 to residue 1129, Fig. 4) (compare lanes 9–12 with lanes 1–4 and lanes 5–8, Fig. 6B) and to a probe derived from intron 5 (from residue 2749 to residue 2826 in Fig. 4), one of the *U14* *snoRNA* coding regions. Densitometric analysis of the autoradiogram illustrated in Figure 6B indicated that the intensity of these bands is 4 or 5 times higher in the DNA samples from HR-1 cells when compared to the corresponding bands in the DNA samples from HA-1 and CHO cells (data not shown). Similar results were obtained with three other independent DNA preparations, each performed in a manner to avoid cross contamination. Overall, these data indicate that the *hsc70/U14* gene may be amplified in the HR-1 cells and that the unit of amplification must be large, since the size of the cross-hybridizing bands in Figure 6B are unchanged and no new hybridizing bands

were detected in the HR-1 DNA after digestion with several restriction enzymes.

The possibility that the *hsc70/U14* gene is amplified in HR-1 cells was further examined by comparing the chromosomal localization of this gene in HA-1 and HR-1 cells. This was accomplished by performing in situ hybridization using the entire insert of clone 19–2a as probe. The genomic insert in this clone is 20 kb, which is about 10 times larger than the corresponding cDNA clone (Ahmad et al 1990). High stringency hybridization conditions were used to minimize cross-hybridization with other *hsp70* genes. The results of these experiments are shown in Figure 2. Two small submetacentric chromosomes with positive signals (similar in size to chromosome 9 in CHO cells; Worton et al 1977) which appear not to be totally homologous according to their banding pattern, are seen in HA-1 metaphase cells (arrowheads, Fig. 2A). In HR-1 metaphase cells (Fig. 2B), the signal is seen on one chromosome which has the same banding pattern as one of the chromosomes that gave a signal in HA-1 cells, (arrowhead, Fig. 2B) and on two additional chromosomes which are not observed in HA-1 cells. One of these two new chromosomes is a large submetacentric chromosome (indicated by an open arrowhead in Fig. 2B) and the other is a small submetacentric chromosome displaying an intense signal in the telomeric region (arrow, Fig. 2B).

Further analysis, the results of which are currently in preparation, indicated that the new large submetacentric chromosome displaying a signal in HR-1 cells may be a rearrangement involving one of the original submetacentrics displaying a signal in HA-1 cells and a larger submetacentric chromosome. The smaller metacentric with the telomeric signal represents the remaining part of one of the original submetacentrics containing the gene (the short arm and a part of the long arm), which was involved in this rearrangement. The telomeric signal in the new submetacentric chromosome found in HR-1 cells was observed to be consistently brighter than those found in the other two chromosomes in these cells, or those found in the parental HA-1 cells. This is particularly noticeable when interphase nuclei are compared as shown in Figure 3. The overall results of the in situ hybridization are consistent with the presence of two single copies of the *hsc70/U14* gene in HA-1 cells and an amplification event in HR-1 cells. These results also confirm that the unit of amplification must be large, as was deduced above from the lack of change in the size of the putatively amplified bands observed in the genomic Southern in Figure 6B.

Altered expression of Hsc70 mRNA and U14 snoRNA in HR-1 cells

The effect of a mild heat shock on the expression of Hsc70 in HA-1 and HR-1 cells was examined next. Total

cellular RNA was isolated from control cells and cells which had been heat shocked at 45°C for 15 min and were then allowed to recover at 37°C for various lengths of time and analyzed on a Northern blot. A single band migrating at 2.7 kb was detected in all of the lanes using a probe made from the 3' non-coding region of the *hsc70* gene (from residue 4340 to residue 4553, Fig. 4) (Fig. 7A). The level of the Hsc70 mRNA increased after 6 h of recovery from the initial heat shock and remained elevated for over 14 h in HR-1 cells, but returned to control levels in HA-1 cells (Fig. 7A, data not shown). As illustrated in Figure 7C, the levels of β -actin mRNA were found not to vary significantly on the same blot, indicating that similar amounts of RNA from each experimental time point were loaded. Similar differential induction patterns of Hsc70 mRNA between HA-1 and HR-1 cells were observed after exposure to either 43°C or 45°C for various lengths of time, exposure to sodium arsenite and exposure to amino acid analogs (data not shown).

The blot was then stripped and rehybridized under the same experimental conditions with a probe made from the insert of pM3, a heat inducible cDNA clone isolated

from Chinese hamster V79 cells (Fornace et al 1989). As described in detail under Materials and Methods, we have identified this cDNA as encoding the major heat-inducible form of the Hsp70 family in Chinese hamster cells (M.-S. Chen and A. Laszlo, unpublished observations). No detectable levels of the Hsp70 mRNA were observed in control, non-heated HA-1 or HR-1 cells, confirming earlier observations made at the level of protein (Laszlo and Li 1985; Ohtsuka and Laszlo 1992). An increase in the levels of mRNA coding for Hsp70 was observed 4 h after the heat treatment, reached a maximum at 6 h, and then decayed gradually in both cell lines (Fig. 7B). The fact that this hybridization pattern is significantly different from that obtained with the Hsc70-specific probe (see above in Fig. 7A), supports our interpretation that under the stringency of the hybridization and washing conditions used, only the heat-inducible Hsp70 mRNA was being detected with this probe. The pattern of hybridization obtained after heat shock is similar to that reported for Hsp70 mRNAs in mouse, human, and other hamster cell lines (Hunt and Morimoto 1985; Hickey et al 1986; Fornace et al 1989; Hunt and Calderwood 1990). A comparison of the relative induction of the Hsp70 mRNA revealed that the heat-induced increase in the abundance of the Hsp70 mRNA is greater in the HA-1 wild-type cells than in the HR-1 cells. For the same heat dose, the opposite pattern was found with the relative induction of the Hsc70 mRNA above (Fig. 7A). These results are consistent with earlier observations made concerning the level of Hsc70 and Hsp70 proteins in these cells under similar experimental conditions: higher levels of Hsc70 and lower levels of Hsp70 were observed in HR-1 cells exposed to a variety of heating regimes (Laszlo and Li 1985; Laszlo 1988a; Ohtsuka and Laszlo 1992).

A smaller mRNA was also detected by the Hsp70-specific probe, but its expression was not induced by heat shock. This mRNA is probably the product of a tissue-specific *hsp70* gene, since it cross-hybridized to a probe generated from the 3' non-coding region of the mouse testis specific *hsp70t* gene (a gift from Dr C. Hunt, Washington University, St Louis University, St Louis) (Zakeri et al 1988; M.-S. Chen and A. Laszlo, unpublished observations). This latter gene is expressed in ovaries (Zakeri et al 1988) and, thus, its expression in the cell lines used in this study probably reflects their ovarian origin (Laszlo and Li 1985).

The *hsc70/U14* gene also contains three copies of the *U14* *snoRNA* gene in introns 5, 6 and 8, respectively (Fig. 4). The effect of heat shock on the levels of *U14* *snoRNA* was examined in the experiment illustrated in Figure 8. HA-1 and HR-1 cells were exposed to 45°C for 15 min and allowed to recover at 37°C for various lengths of time up to 14 h. The levels of Hsc70 mRNA were monitored by hybridization with the non-coding region

Fig. 7 Northern blot analysis of total cellular RNA isolated from control and heat shocked HA-1 and HR-1 cells with probes specific for Hsc70 and Hsp70. Exponentially growing cells were exposed to 45°C for 15 min and allowed to recover at 37°C for 0, 1, 2, 4, 6, 8, 10, 12 and 14 h. Equal amounts of total cellular RNA (5 µg) from control (C) and heated cells were separated on a 1.5% agarose gel containing formaldehyde and blotted onto nitrocellulose. (A) Hybridization with a ^{32} P labeled probe generated from the 3' non-coding region of the *hsc70/U14* gene (from residue 4340 to residue 4553, Fig. 4) under high stringency conditions: (10% dextran-sulfate in 1 M. NaCl, 1% SDS, at 65°C, washed with 0.1 × SSC/0.1% SDS at 65°C for 2 h). The autoradiogram represents an overnight exposure. (B) The same filter was hybridized with a probe made from the insert of clone pM3, a Chinese hamster Hsp70 cDNA clone (see Materials and Methods) using the same hybridization conditions. (C) The same filter was hybridized with a β -actin cDNA to determine relative loading of the different samples.

Fig. 8 A comparison of the induction of Hsc70 mRNA and U14 snoRNA in HA-1 and HR-1 cells. Exponentially growing cells were exposed to 45°C for 15 min and allowed to recover at 37°C for 2, 4, 6, 8, 10, 12 and 14 h. Equal amounts (5 µg) of total cellular RNA isolated from control (C) and heated cells (5 µg) were analyzed exactly as described in Figure 7 for levels of Hsc70 mRNA or electroblotted and challenged with a probe specific for the U14 RNA associated with intron 5 of the *hsc70/U14* gene (residues 2749 to 2826, Fig. 4). The figures represent autoradiograms obtained after a 48-h exposure.

specific probe described above, while the levels of U14 snoRNA were monitored with a probe generated from the U14 snoRNA coding region contained in intron 5 (residues 2749 to 2826 in Fig. 4). The pattern of heat-induced increase in the abundance of Hsc70 mRNA in both HA-1 and HR-1 cells is similar to that observed in the previous experiment (Fig. 7A). There is an increase in the levels of U14 snoRNA in both cell lines which parallels the increase in Hsc70 mRNA under these conditions. Although not clearly supported by this figure, we have found that the abundance of U14 snoRNA is about 2-fold greater in control, non-heated HR-1 cells than that found in the parental HA-1 cells (M.-S. Chen and A. Laszlo, unpublished results). About 2-fold higher levels of Hsc70 mRNA have also been found in HR-1 cells as indicated by in-vitro translation (Laszlo and Li 1985) and molecular hybridization using a rat Hsc70 clone (Laszlo 1988a).

DISCUSSION

The studies presented here were undertaken to characterize the molecular and genetic basis of the heat resistant phenotype of HR-1 cells (Laszlo and Li 1985). Previous studies have indicated that these cells expressed elevated levels of the Hsc70 polypeptide under normal growth conditions, reflecting elevated levels of Hsc70 mRNA as monitored by in vitro translation (Laszlo and Li 1985)

and by hybridization with a rat *hsp70* cDNA probe (Laszlo 1988a). The regulation of the expression of the major heat shock proteins is also altered in these cells, as indicated by the observation that for every stress tested (including heat shock, sodium arsenite and amino acid analogs) there is a larger relative increase in the synthesis and accumulation of Hsc70 and Hsp110, and a smaller relative increase in the synthesis and accumulation of Hsp90 (Laszlo 1988a; Ohtsuka and Laszlo 1992). Here, the molecular cloning of the *hsc70* gene from Chinese hamster cells allowed the demonstration that the heat resistant phenotype of HR-1 cells is associated with the amplification of the Hsc70 structural gene, and revealed novel facts of the regulation of the expression of Hsc70 in hamster cells.

The *hsc70/U14* gene and its homologs in Chinese hamster cells

One of the four genomic independent clones we isolated contained the *hsc70/U14* gene, while the other three contained intron-less coding regions and lacked any known transcriptional regulatory elements within 800 bp of non-coding regions at the 5' end. Since sequencing indicated that these clones contain internal stop codons and frameshifts, they probably represent pseudogenes. Restriction mapping of 28 other independently isolated

genomic clones using full length, 5' end or 3' end probes of the hamster *hsc70* cDNA showed that their genomic DNA fragments were small, indicating that they probably did not contain introns. Since these fragments did not cross-hybridize with pM3, a cDNA clone encoding hamster Hsp70 (Fornace et al 1989; M.-S. Chen and A. Laszlo, unpublished observations), they do not appear to be closely related to *hsp70*. When duplicates of the filters used to screen for the *hsc70/U14* gene were screened with this probe in an effort to isolate the Chinese hamster *hsp70* gene, all the *hsc70/U14* positive clones were Hsp70 negative and vice-versa (M.-S. Chen and A. Laszlo, unpublished results). Southern blot analysis of the Chinese hamster genomic DNA with a probe made from the 5' non-coding region of the *hsc70/U14* gene revealed only one band, except in the samples cut with Bam H1, which hybridized to two bands due to an internal Bam H1 site within the region covered by the probe (Fig. 6B, lanes 4, 8 and 12). Overall, these observations strongly support the notion that there is only one functional copy of the *hsc70/U14* gene in hamster cells, as is the case for rat and human cells.

It is of interest to note that the amino acid sequence of the Hsc70 protein is very highly conserved: there is only one amino acid difference between the human and Chinese hamster gene and between the rat and Chinese hamster gene. This degree of sequence conservation is much higher than that found in the heat inducible members of the Hsp70 family (Hunt and Morimoto 1985; Gupta and Singh 1994). Such high sequence conservation may indicate different functions for the heat inducible and the cognate forms of Hsp70, different selection pressure on these two molecules or a combination of both. Evidence has been presented that Hsc70 and Hsp70 share some biochemical similarities (Brown et al 1993) yet under certain situations play different roles (Walton et al 1994). Furthermore, using monospecific antibodies against Hsp70 and Hsc70, we have found that Hsc70, but not Hsp70, is associated with the nucleolus under normal growing conditions (Laszlo 1992; Laszlo et al, ms in preparation).

A novel feature of the functional vertebrate *hsc70* genes is that three of their introns code for a small nucleolar RNA, called *U14 snoRNA* (Liu and Maxwell 1990; Leverette et al 1992). In mouse cells, it has been demonstrated that in the entire genome, the *U14 snoRNA* coding sequences are only located in these introns of *hsc70* (Leverette et al 1992). This is also the case for the hamster *U14 snoRNA* sequences (M.-S. Chen and A. Laszlo, ms in preparation).

Regulation of the expression of the *hsc70/U14* gene

Alignment of the nucleotide sequences of the promoter region of the rat, human and Chinese hamster *hsc70/U14*

genes indicated that the nucleotide sequence of putative transcriptional control elements are completely conserved (Fig. 5). These include a TATA box, two CAT boxes, and two sets of HSEs. In the case of the latter, not only are the NGAAN-inverted repeats completely conserved, but the whole region containing them is also conserved. In spite of this conservation, the induction of the expression of Hsc70 in both rat and human cell lines by heat shock was found to be weak (Dworniczak and Mirault 1987; Sorger and Pelham 1987). However, under the experimental conditions used in this report, the levels of Hsc70 mRNA of both wild type HA-1 cells and their HR-1 heat-resistant derivatives increased as much as 10-fold following exposure to a mild heat shock (Fig. 7). Similar increases in the levels of Hsc70 mRNA were also detected after exposure of these cell lines to other inducers of the heat shock response, including sodium arsenite and the proline analog L-azetidine-2-carboxylic acid (M.-S. Chen and A. Laszlo, ms in preparation). These observations are consistent with previous reports that the levels of the Hsc70 protein are elevated after heat shock and other stresses in HA-1 and HR-1 cells (Laszlo and Li 1985; Laszlo 1988a, 1988b; Ohtsuka and Laszlo 1992) and after a mild heat shock in CHO cells (Ahmad et al 1990). Similar elevations of the levels of Hsc70 mRNA were likewise observed in other Chinese hamster cell lines exposed to inducers of the heat shock response, including CCL-39 lung cells and OvCa cells derived from an ovarian carcinoma (M.-S. Chen and A. Laszlo, unpublished observations). In all instances, nuclear run-on experiments indicated that heat shock led to an increase in the transcriptional activity of the *hsc70* gene (M.-S. Chen and A. Laszlo, ms in preparation). This apparent differential response of the *hsc70* gene in hamster vs rat and human cell lines might be due to different experimental conditions, or could be a reflection of different regulatory mechanisms acting in different species or even in different cell lines from the same species. It is possible that the induction of Hsc70 in human and rat cell lines was not observed because more moderate heat shocks were used, and because RNA was isolated from cells which had recovered only up to 4 h after the initial heat shock was examined. In our experiments, the increase in the abundance of Hsc70 mRNA was observed 2 h later than the increase in the abundance of Hsp70 mRNA. On one hand, increased levels of the Hsc70 protein were detected in HA-1 and HR-1 cells, but not in HeLa cells subjected to a 15-min heat shock at 45°C and allowed to recover for 24 h, using a monoclonal antibody which is specific for Hsc70, while on the other hand, increases in the levels of Hsp70 protein were detected in both cell lines (A. Hu and A. Laszlo, unpublished observations). This result indicates that the heat shock protocol leading to an elevation in the levels of Hsc70 mRNA in hamster cells did not elicit the same

effects in at least in one human cell line. Further studies will be necessary to decipher the basis for these apparently different responses of the *hsc70* gene to heat shock in hamster and human cell lines.

Genomic Southern (Fig. 6) indicated a 4- to 5-fold increase in the copy number of a unique restriction fragment associated with the *hsc70* gene. On the other hand, the expression of the mRNA coding for Hsc70 and the U14 snoRNA is, at most, only, 2-fold greater in the HR-1 cells, both under normal growth conditions and after exposure to various stresses (Laszlo and Li 1985; Laszlo 1988a; A. Laszlo and M.-S. Chen, unpublished observations). Thus, either not all amplified copies of the *hsc70* gene in the HR-1 cells are functional, or there is some type of compensatory mechanism which regulates the levels of Hsc70 mRNA and protein. The latter notion is supported by the current understanding that the expression of the members of the Hsp70 family is self-regulated (Morimoto 1993) and by results from using cell lines expressing antisense constructs of *hsc70* (Li and Hightower 1995).

The effect of a mild heat shock on the levels of U14 snoRNA was also examined in HA-1 and HR-1 cells. There was an increase in U14 snoRNA levels which paralleled the increase in the Hsc70 message, as would be expected from the location of the *U14 snoRNA* genes in introns 5, 6 and 8 of the gene coding for Hsc70. We could not find any identifiable transcriptional control elements associated with the hamster U14 snoRNA sequences, similar to what has been found with mouse U14 snoRNA (Leverette et al 1992). Furthermore, the *U14 snoRNA* genes found in the introns of the *hsc70* gene are the only copies of this gene in the Chinese hamster genome. Thus, the possibly coordinate parallel increase in the levels of U14 snoRNA and Hsc70 mRNA after heat shock suggests that the expression of the U14 snoRNA is controlled at the level of transcription through the regions which also control the expression of the *hsc70* gene. The observation that the constitutive levels of U14 snoRNA may be elevated in HR-1 cells under normal growth conditions to a degree similar to that found with Hsc70 mRNA is further evidence for the validity of this notion.

Amplification of the *hsc70* gene in HR-1 cells

The presence of elevated levels of Hsc70 mRNA in HR-1 cells under normal growth conditions and after exposure to various stresses in this report could be a consequence of altered regulation of the *hsc70* gene, an increase in its copy number (gene amplification) or some combination of both. Genomic Southern blots with probes specific for Hsc70 indicated that there may be a 4- to 5-fold increase in the DNA coding for Hsc70. In situ hybridization with the whole genomic insert of clone 19-2a which contains

the entire *hsc70/U14* gene, indicated that the HR-1 cells carry a chromosomal rearrangement of the *hsc70/U14* locus in addition to yielding a more intense hybridization signal (Figs 2 and 3). Taken altogether, these results constitute strong evidence that the *hsc70/U14* gene is amplified in the HR-1 cells. This is the first reported instance of the amplification of a major heat shock protein gene in mammalian cells, and this event is associated with increased survival after exposures to elevated temperatures.

Gene amplification is a process that has been studied in certain normal developmental processes (reviewed in Stark et al 1989, 1990) and in the acquisition of resistance of cells in culture to a variety of cytotoxic drugs (reviewed in Schimke 1984; Hamlin et al 1991; Hamlin 1992; Sharma and Schimke 1994). Gene amplification at several loci in Chinese hamster cells which have developed resistance to a variety of chemotherapeutic drugs has been associated with the presence of homogeneously staining regions when chromosomes are visualized at metaphase (Hamlin et al 1991; Sharma and Schimke 1994). Detailed studies of early events in the amplification of the dihydrofolate-reductase gene have demonstrated the involvement of chromosomal aberrations, which included translocations, events involving bridge/breakage/fusion cycles generated from chromosome breakage and terminal fusion or reverse unequal sister chromatid exchanges (Flinthoff et al 1984; Trask and Hamlin 1989). The bridge/breakage/fusion cycles are thought to mediate subsequent amplification steps (Ma et al 1993). Most recently, major fragile sites in the CHO genome have been shown to be involved in amplification of the P glycoprotein gene (Kuo et al 1994).

The selection scheme that was used to generate the HR-1 cells (Laszlo and Li 1985) involved two distinct steps: one prolonged treatment at 43°C (6 h) followed by a 45-min treatment at 45°C. This scheme could have led to the selection of a pre-existing population of cells which were heat resistant or to the induction of gene amplification. Previous attempts to generate heat resistant variants by culturing survivors of prolonged exposures to 45°C alone have not been successful (A. Laszlo and G.C. Li, unpublished observations; G.C. Li, unpublished observations). Thus, it appears that the 43°C selection step was critical in the isolation of the HR-1 cells and we hypothesize that events that took place during this prolonged exposure to a milder hyperthermic treatment could have led to the gene amplification events that we have observed here. Exposure to such a temperature is known to lead to the induction of chromosomal aberrations (Dewey et al 1971) and the inhibition of DNA synthesis (reviewed in Laszlo 1992). The results of the in-situ hybridization experiments (Fig. 2) indicated that a translocation of the original site of the locus was probably

involved in the amplification event, consistent with similar observations describing early events in drug resistant cells undergoing gene amplification (Flinthoff et al 1984; Hamlin et al 1991). Thus, it appears that the HR-1 cells display cytological features that are similar, if not identical, to those that have been found in the early stages of gene amplification that have been associated with the establishment of drug resistant phenotypes. This supports our hypothesis that the heat selection scheme we used induced the amplification of the *hsc70* gene. The lack of obvious alterations in the size of the putatively amplified fragment is consistent with the observation that large regions are involved in amplification events, which is also supported by the results of the in situ hybridization. In addition, selection for heat resistance with heat appears to be similar to selection with drugs such as methotrexate, since both these agents inhibit DNA synthesis and cause chromosomal aberrations. Many processes which interfere with DNA replication also increase the frequency of gene amplification (Stark et al 1989, 1990; Sharma and Schimke 1994).

The possible induction of gene amplification by hyperthermia is a novel finding. In addition to delineating yet another perturbation which can lead to gene amplification, this finding may have implications for the clinical use of hyperthermia in that increased sensitivity to chemotherapeutic drugs is one of the salient effects of exposure of mammalian cells to elevated temperatures (Herman and Teicher 1994). Combined hyperthermiachemotherapeutic protocols are being used at several institutions. Our results indicate that drug resistant cell populations may arise during such protocols and further investigations of the process of gene amplification by exposure to elevated temperatures is warranted.

Molecular basis of heat resistance in HR-1 cells

In this report we have demonstrated that the heat resistant phenotype of HR-1 cells, which is associated with the overexpression of Hsc70 at normal growth temperatures (Laszlo and Li 1985; Laszlo 1988a; Ohtsuka and Laszlo 1992) is due to the amplification of the gene that codes for Hsc70. Our studies presented here also indicate that there is no alteration in the endogenous expression of Hsp70 in the HR-1 cells. Thus, the overexpression of Hsc70 alone can confer permanent heat resistance, as has been shown for Hsp70 and Hsp27 (Landry et al 1989; Li et al 1991, 1992). Although Hsp70 and Hsc70 are very similar in their amino acid sequence (Morimoto et al 1994), evidence has been presented that they may have different cellular functions under some experimental conditions (Laszlo 1992; Walton et al 1994). Nevertheless, it appears that these two closely related molecules can both confer a heat resistant phenotype. However, the mechan-

isms involved are not clear, which is a reflection of the lack of understanding of the mechanisms of heat induced cell killing (Laszlo 1992).

A significant difference between *hsc70* and *hsp70* is that the former contains introns while the latter does not. The discovery that three of the introns of *hsc70* code for U14 snoRNA, and that this organization is found in all vertebrate cells examined so far (Morimoto et al 1994), has potentially significant implications for the cellular response to elevated temperatures. Although the function of U14 snoRNA in mammalian cells is not known at the present time, it is an essential gene in yeast (Li et al 1990; Li and Fournier 1992), where it is involved in ribosomal biogenesis (Li and Fournier 1992). Since a hybrid yeast-mouse U14 snoRNA can perform this essential function in the yeast cell (Li et al 1990), it is likely the vertebrate U14 snoRNA performs a similar function. Alterations of the structure and function of the nucleolus and in ribosomal biogenesis are some of the major documented effects of exposure to elevated temperatures (reviewed in Nover 1991; Laszlo 1992). We have found that the expression of U14 snoRNA may also be elevated in HR-1 cells and that its levels are elevated after heat shock. Thus, it is formally possible that elevated levels of U14 snoRNA could play a part in the heat resistant phenotype of HR-1 cells, perhaps by being involved in the restoration of heat-induced alterations in ribosomal biogenesis. This possibility is currently under investigation in our laboratory.

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