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Polymorphic analysis of the three MHC-linked *HSP70* genes

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Abstract. Three genes encoding members of the M_r 70 000 heat shock protein family (HSP70) are known to lie in the class III region of the human major histocompatibility complex. In order to determine whether these genes or their protein products exhibit any polymorphism the three genes have been specifically amplified from genomic DNA and sequenced. The *HSP70-1* and *HSP70-2* genes encode the major heat-inducible HSP70. A comparison of the nucleotide sequences of these genes from *B8, SC01, DR3, B18, F1C30, DR3*, and *B7, SC30, DR2* haplotypes has revealed only very limited sequence variation which is not associated with any amino acid polymorphism. The *HSP70-Hom* gene encodes a protein that is highly related to *HSP70-1*, but which is not heat-inducible. Nucleotide sequence analysis of this gene from different haplotypes has revealed a Met→Thr amino acid substitution at residue 493 in a number of the haplotypes tested. This variable amino acid lies in the proposed peptide-binding site of the HSP70-Hom protein.

Introduction

The M_r 70 000 heat shock proteins (HSP70) are encoded by a multigene family in human. The members of this protein family are highly structurally related, but differ in their cellular localisation and their patterns of expression in response to metabolic stress (reviewed by Morimoto and Milarski 1990; Morimoto et al. 1990). Three genes encoding members of the HSP70 family are located in the class III region of the human major histocompatibility complex (MHC), 92 kilobases (kb) telomeric of the complement *C2* locus (Sargent et al. 1989; Milner and Campbell 1990). These have been defined as *HSP70-1*, *HSP70-2*, and *HSP70-Hom*. The *HSP70-1* and *HSP70-2* genes have been shown to encode an identical protein product which is the major heat-inducible HSP70 protein (Hunt and Morimoto 1985). The *HSP70-Hom* gene encodes a previously undescribed member of the HSP70 protein family which shares 90% sequence identity with HSP70-1.

The use of locus specific probes, isolated from the 3'-untranslated regions (UT) of the MHC-linked *HSP70* genes, has shown that the *HSP70-1* and *HSP70-2* genes are both expressed at very high levels in cells heat shocked at 42 °C (Milner and Campbell 1990). *HSP70-1* is also constitutively expressed at low levels. Sequence differences in the 5'-flanking regions of the *HSP70-1* and *-2* genes suggest that they may be differentially regulated in response to stress factors other than heat shock. The *HSP70-Hom* gene is constitutively expressed at low levels, but is not induced by heat shock.

The identification of *HSP70* genes in the class III region is of particular interest with regard to the involvement of the MHC in disease susceptibility since members of the HSP70 protein family are known to be involved in the immune response. For example, the HSP70 proteins are major antigens in many bacterial and parasitic infections (reviewed by Young et al. 1990) and both antibodies and T cells directed against autologous stress proteins have been identified in patients suffering from a number of autoimmune diseases (Winfield and Jarjour 1991). In addition, there are a number of potential roles for the HSP70 proteins in antigen processing and presentation (reviewed by Pierce et al. 1991). A member of the rat HSP70 family, prp73, has been shown to bind cytosolic proteins which contain a particular peptide motif and thus mediate their translocation to the lysosomes for degradation (Chiang et al. 1989; Dice 1991). A mouse HSP70 protein family member, PBP72/74, has been shown to participate in the binding of antigenic peptides to class II molecules (VanBuskirk et al. 1991). There is only very limited sequence data available for prp73 and PBP72/74 so their similarity to HSP70-Hom or to the heat-inducible HSP70-1 cannot be determined. However, it is interesting

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to note that PBP72/74, like HSP70-Hom, is not heat-inducible.

Southern blot analysis has shown that there are no gross insertions or deletions associated with the MHC-linked *HSP70* genes (unpublished data). However, sequence variation in these genes between haplotypes, resulting in altered expression or protein activity, could be a factor in determining susceptibility to disease. Nucleotide sequence differences in the 5′- and 3′-UT regions of the *HSP70* genes have allowed us to specifically amplify the MHC-linked *HSP70* genes by polymerase chain reaction (PCR) and hence to investigate the presence of sequence variation in these genes between different HLA haplotypes.

Materials and methods

Sources of genomic DNA. Genomic DNA was prepared from HLA homozygous transformed B lymphoblastoid cell lines grown in tissue culture. Three cell lines from the 10th International Histocompatibility Workshop (IHW) were used. These were a *B8, SC01, DR3* haplotype (10th IHW number 9086), a *B18, F1C30, DR3* insulin dependent diabetes mellitus (IDDM)-associated haplotype (10th IHW number 9018) and a *B35, SC31, DR11* haplotype (10th IHW number 9041). A *B8, SC01, DR3, SLE*-associated haplotype (*1518*, provided by Professor R. Dawkins, Sir Charles Gairdner Hospital, Perth, Australia) was also used.

Amplification of the HSP70 genes by PCR. The coding sequences of the MHC-linked *HSP70* genes and the 5′-flanking and UT regions of *HSP70-1* and *-2* were amplified from genomic DNA using sequence specific oligonucleotide primers (Table 1). The PCR reaction mixtures contained: 500 ng of genomic DNA; 200 μM each of dATP, dCTP, dGTP, and dTTP; 1 μM each primer; and 1 unit of *Taq* DNA polymerase in 1 × PCR buffer provided by the manufacturer (Promega, Madison, WI). Amplification was accomplished by 30 cycles of incubation at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 3 min followed by a final incubation at 72 °C for 10 min.

Cloning of PCR products. The products of PCR amplification of the *HSP70* genes were cloned into pBluescript vectors for sequence analysis. Prior to cloning the DNA was first phosphorylated and then digested with *Bam* HI. To achieve phosphorylation, the DNA (≤ 50 pmol 5′ ends) was incubated with 10 units T4 polynucleotide kinase (Boehringer Mannheim, Lewes, UK) in 18 mM $MgCl_2$/5 mM DTT/50 mM imidazole-HCl (pH 6.4)/6% (w/v) PEG 4000/50 pmol rATP. Reactions were incubated for 30 min at 37 °C and then stopped by the addition of EDTA (pH 7.4) to a final concentration of 0.125 M. The DNA was recovered by extraction with chloroform/isopropanol (24:1, v/v) and three rounds of ethanol precipitation. The phosphorylated DNA was digested with *Bam* HI and then purified from LGT agarose. The PCR products were cloned into *Bam* HI/*Hinc* II-cut pBluescript KS^+ or SK^+ vectors.

Nucleotide sequence analysis. Single-stranded DNA was recovered from the pBluescript clones using the helper phage M13K07 in the presence of kanamycin. Nucleotide sequencing was carried out by the dideoxy nucleotide chain termination method using Sequenase (US Biochemicals, Cleveland, OH). The clones containing the *HSP70-1* and *-2* coding regions were sequenced in one orientation using the M13 universal primer, the specific oligonucleotide primers OL2-OL7 and OL10 (*HSP70-1*) or OL12 (*HSP70-2*) as shown in Table 1. The *HSP70-Hom* coding region was similarly sequenced using the M13 universal primer and the specific oligonucleotides OLH1-OLH3 and OLH11-OLH13 (Table 1). The 5′-flanking and UT regions of *HSP70-1* and *HSP70-2* were sequenced in both orientations using the M13 universal primer.

Results

Amplification and cloning. The original sequences of the *HSP70-1*, *HSP70-2*, and *HSP70-Hom* genes were derived from an *A2, B7*, SC30, *DR2* haplotype (Milner and Campbell 1990). There are significant sequence differences between the 5′- and 3′-UT regions of the *HSP70-1*, *-2* and *-Hom* genes. These regions are also quite distinct from the corresponding regions of other published *HSP70* sequences. Thus it was possible to design oligonucleotide primers to allow the specific amplification of the three

Table 1. Oligonucleotides used for sequence analysis and PCR amplification of the *HSP70* genes.

	HSP70-1	*HSP70-2*	*HSP70-Hom*	
Sequence*	OL2 (468–489)	GTCGGACATGAAGCACTGGC	OLH1 (1822–1841)	CACTTTATGAAGGCATTGAC
	OL3 (762–781)	CTACGGCCTGGACAGAACGG	OLH2 (1207–1226)	CTGTTGTACAAGCAGATATG
	OL4 (1084–1101)	GGCATCGACTTCTACACG	OLH3 (2717–2736)	CAACTGGCAGAGAAAGATG
	OL5 (1342–1360)	GCAGGCGGCCATCCTGATGG	OLH11 (1497–1515)	GCTGCTGCCATTGCCTATG
	OL6 (1620–1639)	CAGGGGCGTGCCCCAGATCG	OLH12 (2111–2132)	GGACAAGTCTGAGAAGGTACAG
	OL7 (1886–1904)	GGCTCAAGGGCAAGATCAG	OLH13 (2423–2411)	CAATGTCACAGCCACGGAC
	OL10 (2120–2137)	CCCACCATTGAGGAGGTAG	OL12 (2118–2135)	CCTACCATTGAGGAGGTGG
PCR*				
Sense	OL18 (120–138)	TCCGGCGTCCGGAAGGACC	OL19 (128–147)	TCCGAAGGACTGAGCTCTTG
	OLH10 (924–959)	CCGGATCCCATAGGCCTCAGAGAACC		
	OL20 (−275–257)	CGCCATGGAGACCAACACC	OL22 (−232–214)	CTCTCAGGGCCCCTGTCC
Antisense	OL9 (2253–2272)	GGCAAGTTCAGTACTTCACC	OL14 (2184–2203)	CAGCAAAGTCCTTGAGTCCC
	OLH8 (2968–2988)	GTAACTTAGATTCAGGTCTGG		
	OL21 (178–196)	CGGCTCGGCTCTGAGATTG	OL23 (188–205)	CTGCTCTGTGGGCTCCGC

* Numbers in brackets refer to the positions of the oligonucleotides in the relevant sequences (Milner and Campbell 1990).
All oligonucleotide sequences are written 5′ to 3′ from left to right except the antisense PCR primers which are 5′ to 3′ from right to left.

MHC-linked *HSP70* genes by PCR (Table 1). The coding sequences of these genes were amplified using the primers OL9 and OL18 (*HSP70-1*), OL19, and OL14 (*HSP70-2*), and OLH10 and OLH8 (*HSP70-Hom*). In all cases unique products of ~2 kb were obtained. The 5'-flanking and UT regions of *HSP70-1* and *-2* were amplified using the primers OL20 and OL21 (*HSP70-1*) and OL22 and OL23 (*HSP70-2*). Products of ~450 base pairs (bp) were obtained in each case.

The coding sequences of the three MHC-linked *HSP70* genes and the 5'- flanking and untranslated regions of *HSP70-1* and *-2* were specifically amplified from genomic DNA prepared from the cell lines 9086, 9018, and 1518 (see Materials and methods). The 5'-flanking and UT regions of *HSP70-1* and *-2* were also amplified from the cell line 9041. The 5'-flanking and UT regions of *HSP70-Hom* have been excluded from this analysis since the transcriptional start site of this gene has not yet been identified and no functional regulatory elements have been defined.

The PCR products corresponding to the three MHC-linked *HSP70* genes were phosphorylated and digested with *Bam* HI prior to cloning. Each of the PCR products had a unique restriction site for *Bam* HI close to one end and this allowed their directional cloning into pBluescript vectors.

Nucleotide sequence analysis. Single-stranded DNA was recovered from the pBluescript clones for sequence analysis. At least three independent clones corresponding to each PCR product were sequenced to take into account the possibility of PCR errors (Ennis et al. 1990).

The sequence analysis of the *HSP70* genes from the HLA homozygous cell lines described above revealed eight instances where the cloned PCR products consistently differed at a particular nucleotide position compared to the original *B7, SC30, DR2* sequence (Table 2).

In the *HSP70-1* gene nucleotide positions -110, 190, and 438 are polymorphic. Position 438 lies in the coding region, but the C→T transition observed in the *B7, SC30,* DR2 haplotype compared to the other haplotypes sequenced is a silent mutation. Nucleotide -110 lies in the 5'-flanking region of the *HSP70-1* gene just 3 bp upstream of the heat shock element (underlined with consensus residues shown in bold) which is essential for the heat inducibility of the *HSP70-1* gene (Morimoto and Milarski 1990):

AAAACCCTGGAATATTCCCG
C
−110

In the *HSP70-2* gene nucleotide positions 145, 1267, and 2074 are polymorphic. Of these positions 1267 and 2074 lie in the coding region, but correspond to silent mutations. The A→G transition at position 1267 lies in a polymorphic *Pst* I site (Goate et al. 1987; Milner and Campbell 1990).

Two polymorphic nucleotides have been identified in the coding sequence of the *HSP70-Hom* gene. The first of these, at position 1097, is a silent mutation. However, there is a T→C transition at position 2437 in the B7, *SC30, DR2* haplotype. This corresponds to a Met→Thr amino acid substitution at position 493. The nucleotide difference at position 2437 lies within an *Nco* I restriction site (CCATGG). It has therefore been possible to assess the distribution of Thr and Met at amino acid position 493 in a range of different haplotypes.

Characterization of the Nco I polymorphism in HSP70-Hom. The *HSP70-Hom* gene was amplified from genomic DNA derived from 62 cell lines of known HLA specificities, using the primers OLH10 and OLH8. The PCR products were digested with *Nco* I. The presence of an

Table 2. Comparison of the *HSP70* gene sequences from HLA homozygous cell lines.

HLA type	Nucleotide position*							
	HSP70-1			*HSP70-2*			*HSP70-Hom*	
	5' Flanking	5' UTR	Coding	5' UTR	Coding		Coding	
	−110	190	438	145	1267	2074	1097	2437
B7, DR2	A	G	T	T	A	C	C	C
B8, DR3	C	C	C	C	G	C	C	T
B8, DR3 (SLE)	C	C	C	T	G	C	T	T
B18, DR3 (IDDM)	C	C	C	T	G	G	C	T
B35, DR5	A	C	N. D.	T	N. D.	N. D.	N. D.	N. D.

* Nucleotide position is based on the sequences in Milner and Campbell (1990).
N. D. = not determined.

Table 3. Distribution of the HSP70-Hom *Nco* I polymorphism.

Workshop number	Cell line	HLA type				*Nco* I site*
		A	Cw	B	DR	
9080	EHM	3	4	35	1	+
	IBW4	3	4	35	1	+
9005	HOM2	3	1	27	1	+
9078	PMG075	3/33	8	65	1	+
9003	KAS116	24	ND	51	1	+
	ICE5	2	7	7	2	−
	PGF	3	ND	7	15	−
9014	MGAR	26	7	8	15	−
	BBF	1	6	37	15	−
9011	E4181324	1	ND	52	15	+
9015	WT24	2	2	27	16	+/−
	WT18	2	2	27	16	+
9009	KAS011	1	6	37	16	+
9016	RML	2	ND	51	16	+
9084	CALEGARO	2	2	61	16	+
9007	DEM	2	6	57	4/16	−
9086	I0541265	1	7	8	3	+
	STEINLIN	1	7	8	3	+
	1518	1	ND	8	3	+
	AHB	1	7	8	17	+
	WT49	2	ND	17	17	+
9085	EJ32B	30	5	18	3	+
9018	L0081785	3/24	5	18	3	+
	RSHD	30/68	2	42	18	+
9033	BM14	3	7	7	4	+
9025	DEU	31	4	35	4	+
9092	BM92	25	1	51	4	+
9107	LKT3	24	1	54	4	+
	MT14B	31	10	60	4	+
9029	WT51	23	8	65	4	+
	KT9	24/11	1/3	59/60	4	+
9041	J0528239	1	4	35	11	+
	FPF	1	4	35	11	+
9043	BM21	1	ND	41	11	+
9040	BM15	1	7	49	11	+
9089	BOB	24	ND	51	11	+
	BRIP	24	ND	51	11	+
9036	SPOH	2	5	44	11	+
9065	HHKB	3	7	7	13	−
9055	H0301	3	8	14	13	+
	WT46	32	5	44	13	+
9053	HOR	33	ND	44	13	+
9058	OMW	2	ND	45	13	+
9059	SLE005	2	10	60	13	+
9097	EMJ	2/3	10	60	13	+
	APD	1	ND	60	13a	+
9060	CB6B	1	9	62	13	+
9056	KOSE	2	ND	35	13/14	+
	LZL	2	9	62	14	+
	AZL	2	3	62	14a	+
9048	LBF	30	6	13	7	+
9046	BH	2	6	13	7	+
9094	CF996	2/3	8	14	7	+
9047	PLH	3	6	47	7	−
9106	MANIKA	3	6	50	7	+/−
	MANN	29	ND	44	7	+
9051	PITOUT	29	ND	44	7	+
9049	IBW9	33	8	65	7	+
9100	OLL	31	11	62	8	+
9068	BM9	2	4	35	8	+
9076	T7526	2	11	46	9	−
9077	T7527	2	11	46	9/12	−

Workshop numbers are from the 10th International Histocompatibility Workshop.
* + Denotes the presence of an *Nco* I site at nucleotide position 2434.
− Denotes the absence of an *Nco* I site at nucleotide position 2434.
ND = not determined.

Nco I site (i.e., a T at nucleotide position 2437) was indicated by the cleavage of the ~2 kb amplified product to yield fragments of 1495 bp and 550 bp (Fig. 1).

This analysis showed the absence of an *Nco* I site in just nine out of the 62 cell lines characterized (Table 3) which represent at least 55 different HLA haplotypes. In particular the absence of an *Nco* I site at position 2434 appears to be associated with *DRw15* haplotypes and was observed in three out of the four *DRw15* haplotypes tested (in addition to the *DR2* haplotype, *ICE5*). However, in cell line 9011 (*B52, DRw15*) cleavage of the *HSP70-Hom* gene by *Nco* I indicates that there is a T at position 2437. Nine DRw13 cell lines were tested of which one (9065, *B7, DRw13*) was shown not to have an *Nco* I site at position 2434. Similarly one out of the eight *DR7* haplotypes tested (cell line 9047, B47 , DR7) lacks an *Nco* I site at this position.

When the *HSP70-Hom* amplified products from the HLA homozygous cell lines 9015 (*A2, Cw2, B27, DRw16*) and 9106 (*A3, Cw6, B50, DR7*) were digested with *Nco* I only ~50% cleavage was observed. This suggests that althought these cell lines are HLA homozygous they are not homozygous for the *HSP70-Hom* gene.

Discussion

The presence of genes encoding members of the HSP70 family in the class III region of the human MHC suggested a possible role for these proteins in susceptibility to autoimmune disease. With this in mind we set out to investigate the existence of sequence variation in the MHC-linked *HSP70* genes between different HLA haplotypes. The analysis described here suggests that the level of sequence polymorphism in these genes is very low. This is perhaps not surprising at least in the case of the *HSP70-1* and *-2* genes since these encode a protein that performs essential functions in the cell (Morimoto and Milarski 1990).

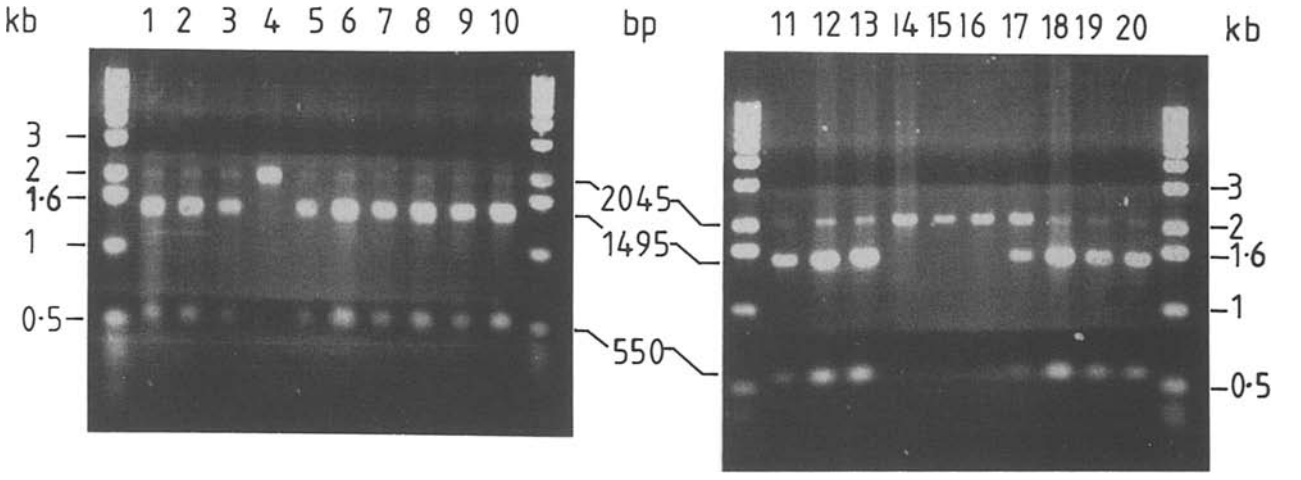

Fig. 1. This figure shows the results of *Nco* I digestion of the HSP70-Hom PCR products from a selection of the cell lines tested. The samples shown are from the cell lines: 1) BM92; 2) J0528239; 3) EMJ; 4) HHKB; 5) SLE005; 6) MANN; 7) PITOUT; 8) CF996; 9) AHB; 10) RSHD; 11) EHM; 12) IBW4; 13) KAS116; 14) ICE5; 15) BBF; 16) MGAR; 17) WT24; 18) RML; 19) CALEGARO; 20) I0541265. Digested samples were fractionated on 0.8% agarose gels. Size markers are shown at the *side* of each gel and fragment sizes are indicated in the *centre* of the figure.

No amino acid sequence differences were observed in the protein product of the *HSP70-1* and *-2* genes in the haplotypes investigated here campared to the *B7, SC30, DR2* haplotype (Milner and Campbell 1990). In all the haplotypes we have studied the sequences of *HSP70-1* and *HSP70-2* differ from the original *HSP70-1* sequence (haplotype not known) reported by Hunt and Morimoto (1985) at amino acid positions 7, 371, and 469. It is not clear whether these differences represent allelic polymorphism in the *HSP70-1* gene.

A single A→C transversion was identified in the 5′-UT region of *HSP70-1* at position −110. However, this does not segregate with those disease associated haplotypes analyzed here. In addition there is a C at the corresponding position in the *HSP70-2* gene which is known to be highly heat inducible. Therefore, it seems unlikely that this polymorphism is associated with any variation in the expression of *HSP70-1* in response to heat shock. It is possible that the variable nucleotide position may lie within a sequence element important for the control of expression of *HSP70-1* in response to some other stress factor.

The members of the HSP70 protein family are known to interact with damaged or denatured proteins and to be involved in the folding, unfolding and translocation of proteins within the cell (reviewed by Langer and Neupert 1991). The constitutively expressed HSC70 has been shown to bind a wide variety of synthetic peptides (Flynn et al. 1989). The release of bound protein from HSP70 family proteins is dependent on ATP hydrolysis. An N-terminal M_r 44000 fragment of HSC70 has been shown to possess ATPase activity (Chappell et al. 1987). This region is highly conserved in all members of the HSP70 family. The role of peptide binding has been attributed to a 170 amino acid region adjacent to the ATPase domain (Chappell et al. 1987; Rippmann et al. 1991). It has recently been proposed that the protein/peptide binding domain of the HSP70 proteins has a structure highly related to that of the HLA class I peptide binding domain (Flajnik et al. 1991; Rippmann et al. 1991). The level of sequence similarity between these protein domains is very low, but the predicted secondary structures for the peptide-binding domains of HSP70-1 and HSC70 are highly related to the crystal structure of the peptide binding domain in HLA-A2 (Bjorkman et al. 1987). A structure for the peptide binding region of HSP70-1 (amino acids 387–557) has therefore been modelled on the basis of the HLA-A2 structure (Rippmann et al. 1991).

The polymorphic amino acid in the HSP70-Hom protein (residue 493) lies within the HSP70 peptide-binding domain. In the structural model for this region proposed by Rippmann and co-workers (1991) amino acid 493 lies on one of the β-sheets which form the floor of the peptide-binding groove. The corresponding position in the HLA-A structure (residue 114) is highly polymorphic (Parham et al. 1988) and lies in one of the six pockets of the peptide-binding groove which determine binding specificity (Madden et al. 1991; Saper et al. 1991). If the HSP70 structural model is correct a Met (non-polar, hydrophobic)→Thr (polar, neutral) substitution at amino acid 493 could be associated with variation in the peptide-binding specificity of HSP70-Hom between haplotypes.

A member of the mouse HSP70 protein family has recently been described that is expressed specifically in spermatids (germ line cells in the haploid phase) (Matsumoto and Fujimoto 1990). The gene encoding this protein, *hsc70t*, has been shown to map to the mouse *H-2* complex. Antisera raised against *hsc70t* have been

used to screen an expression library prepared from human testes and a cDNA corresponding to the human equivalent of *hsc70t* (defined as *hum70t*) has been isolated (Fujimoto et al. 1992). Sequence analysis has shown that *hum70t* is identical to *HSP70-Hom*. The implication that *HSP70-Hom* may be expressed at high levels in spermatids is particularly interesting since the autoimmune condition orchitis is known to be associated with the region of the human MHC to which *HSP70-Hom* maps (Teuscher et al. 1990; Snoek et al. 1991). *HSP70-Hom* may be involved in the presentation of a sperm-specific autoantigen or could itself be the autoantigen.

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