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Original Article

Expression of paternal and maternal mitochondrial HSP70 family, hsc74, in preimplantation mouse embryos

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ABSTRACT We have investigated the regulation of gene expression of a novel mitochondrial HSP70 family, hsc74 in preimplantation mouse embryos. We used a monoclonal antibody, anti-CSA, which reacts with only one of strain variants of the hsc74. By immunostaining with anti-CSA antibody, the hsc74 protein was constitutively detected in C3H embryos from 1-cell to blastocyst stage, but no signals were detectable in C57BL/6 embryos. To know the timing of paternal genome expression, we examined the expression of hsc74 in (C57BL/6 X C3H)F1 embryos. No positive signals were detectable in embryos before 8-cell stage. In early 8-cell stage weakly positive signals appeared in the peripheral region of the blastomeres. From late 8-cell stage, the protein was intensively detectable and was persistently expressed in all types of cells. We have also applied a sensitive methodology to distinguish genetic variants of hsc74 from C3H and C57BL/6 by reverse transcription polymerase chain reaction followed by single strand conformation polymorphism analysis. In (C57BL/6 X C3H)F1 embryos, the paternal transcripts were first detected in 4-cell embryos, while the maternal transcripts were constantly detectable. These results indicate that the transcripts and proteins of hsc74 were derived only from the maternal gene from 1-cell to 4-cell stages, and that from 4-cell stage the paternal gene is also transcribed, and the significant increase of the paternally derived protein occurred around late 8-cell stage.

KEY WORDS: *HSP70 family, mitochondria, chaperone, embryonic genome, mouse early embryo*

Introduction

In 1988, Kusakabe *et al.* reported a mouse antibody against C3H strain specific antigen (CSA), which positively reacted with C3H cells, but not with BALB/c or C57BL/6 (B6) cells. This anti-CSA antibody was obtained in an attempt to establish a suitable cell marker system which can identify the cell origin in tissue recombinant experiments and experimental chimeric mice for studies of cell-cell interactions. An immunohistological analysis revealed that the CSAs do exist in cytoplasm of all types of cells in various tissues of C3H mice. The results of Western blot analysis indicated that an approximate relative molecular mass of the CSA was $72/74 \times 10^3$. Since then, the anti-CSA antibody has been used as a strain specific immunohistological cell marker on the sections of chimeric mice to identify the cells derived from one component strain (Kusakabe *et al.*, 1988; Lee *et al.*, 1991; Yoshiki *et al.*, 1991; Noguchi *et al.*, 1993; Yoshiki *et al.*, 1993a,b; Tatematsu *et al.*, 1994).

We have recently cloned cDNA of the antigenic protein from the kidney cDNA library of C3H mouse by using monoclonal anti-CSA antibody (Michikawa *et al.*, 1993a), and found that the CSA is the same protein as peptide-binding protein72/74 (PBP72/74) (Domanico *et al.*, 1993) and mortalin (Wadhwa *et al.*, 1993a), a novel member of the HSP70 family . The cDNA-derived sequence of CSA indicates that this molecule is initially synthesized as a polypeptide of 679 amino acids with a calculated molecular mass of 73,527 Da. By Northern blot, CSA mRNA can be detected in both CSA-positive and negative strains. The anti-CSA antibody, therefore, recognizes only one of these variants. The deduced amino acid sequences revealed that this molecule structurally consists of three domains: N-terminal transit-peptides, an ATPase domain and substrate-recognition domain in C-terminal. The genetic variation responsible for the CSA antigenicity occurred in the substrate recognition domain of the CSA by an exchange of one amino acid (Arg578), Met in BALB/c and B6 to Arg in C3H (Michikawa *et al.*, 1993a).

The subcellular localization of the CSA was clearly demonstrated immunocytologically as in mitochondria (Michikawa *et al.*, 1993a). An analysis of the genomic organization of the CSA gene revealed that the CSA gene contained 17 exons interrupted

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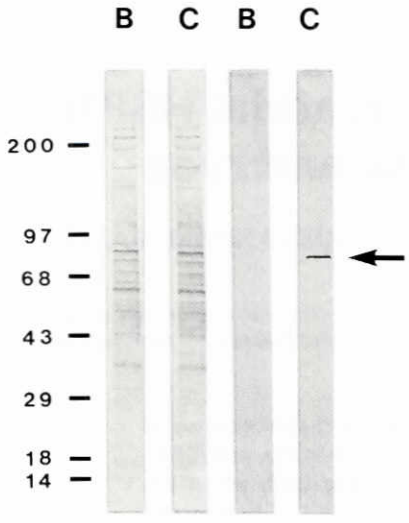

Fig. 1. Demonstration of anti-CSA Mab specificity. Colloidal gold staining (left) and Western blot analysis (right) of B6 (lane B) and C3H (lane C) spleen lymphocyte extracts. *10 μg of protein was loaded on each lane. The result was confirmed in 4 separate experiments. Relative molecular size markers are indicated on the left. An arrow shows the 72 x 10^3 Mr position of hsc74.*

by 16 introns (Michikawa *et al.*, 1993b). Two dimeric repeats of the consensus sequence of the heat-shock element are present in the 5'-flanking region of the CSA gene. Moreover, the first intron is present within the amino-terminal leader sequence, the pattern of which is similar to that of cytochrome c1 located in the mitochondria (Suzuki *et al.*, 1989). Several other mitochondrial stress proteins, grp75, hsp58, and p71, have been identified in mammalian tissues and cultured cells (Mizzen *et al.*, 1989). However, the functions of these proteins are not well understood.

Stress protein families, including hsp60, hsp70 and hsp90, are now collectively known as chaperones, which recognize and stabilize partially folded intermediates during polypeptide folding, assembly and disassembly (reviewed by Gething and Sambrook, 1992). Some of the stress proteins are heat inducible, but other family members are expressed constitutively without any stress. As far as we examined it, the CSA is a non-heat inducible protein that belongs to the HSP70 family. For convenience in the present study, we designated the antigenic protein specific for C3H strain mouse (C3H strain-specific antigen, CSA) as mitochondrial hsc74, and its genetic variants as $hsc74^a$ of C3H and $hsc74^b$ of B6 or BALB/c.

As shown in previous studies (Kusakabe *et al.*, 1988; Michikawa *et al.*, 1993a; Yoshiki *et al.*, 1993b), $hsc74^a$ is expressed in all types of cells in C3H mouse from embryonic periods to adulthood. However, the expression of the hsc74 during preimplantation embryonic period has not been investigated. We present here an analysis of the expression of the hsc74 and its mRNA during preimplantation embryonic development. We have exploited a novel system to distinguish the paternal and maternal gene transcripts by using the reverse transcription polymerase chain reaction (RT-PCR) to amplify the region responsible for the genetic variation of the hsc74, followed by single-strand conformation polymorphism (SSCP) analysis (Hayashi, 1992) in the F1 hybrid embryos derived from B6 eggs fertilized by C3H sperm. In this strain combination we could also successfully detect when the paternal gene product, $hsc74^a$, appeared in the preimplantation embryos by using anti-CSA antibody. Thus, we could assess the timing of embryonic gene activation by detecting the expression of *hsc74* gene in early mouse embryos.

Results

Specificity of the mouse monoclonal anti-CSA antibody

In order to examine the strain specificity of the anti-CSA Mab and the relative molecular mass of antigenic protein, spleen lymphocytes isolated from C3H mice were analyzed by SDS-PAGE and Western blotting. As shown in Fig. 1, one major band was consistently observed corresponding to 72x10^3 Mr in C3H. No bands were observed in control lymphocyte extracts from B6 mouse strain. A band of identical appearance and intensity was seen in the C3H extracts homogenized without Nonidet P-40 (data not shown). Streptavidin/agarose-biotinylated anti-CSA Mab affinity chromatography also revealed an identical band of 72x10^3 Mr (data not shown).

Expression of hsc74 in preimplantation embryos in vivo and in vitro

To examine the expression of hsc74 during the early development, we have fixed and immunostained naturally fertilized embryos of C3H, B6 and (B6 X C3H)F1.

Positive granular stainings were detected in C3H embryos of 1-cell stages at 10 and 18 h post-ovulation (Fig. 2A and B, respectively). At these times, we could observe fertilized eggs with swelling of a part of the egg cytoplasm, the presumptive site of sperm head fusion (Fig. 2A), extrusion of the second polar body (Fig. 2A) and formation of male and female pronuclei (Fig. 2B). Strong signals were detected even in the second polar body (Fig. 2B). Bright granular staining signals were also observed in cytoplasm of every blastomere or cell in C3H embryos through further developmental stages such as 2-cell, 4-cell, 8-cell, morula/blastocyst stages (Fig. 2C,D,E,F, respectively). In contrast, as shown in Fig. 2G,H and I, B6 embryos were negative with anti-CSA Mab from 1-cell stage to blastocyst stage, as were adults derived from this strain .

B6 eggs fertilized with C3H sperm were examined to know the time when paternal gene expression occurs. At 10 h post-ovulation, positive signals by anti-CSA Mab were not observed (Fig. 3A). However, before completion of fertilization, positive signals were detected in the midpiece of the C3H sperms that were attaching on the surface of the eggs (Fig. 3B). We could not detect any CSA positive stainings in embryos at 1-cell (late pronuclear), 2-cell and 4-cell stage embryos (Fig. 3C). In early 8-cell stage at 58 h post-ovulation, weakly positive stainings were observed (Fig. 3D). The intense CSA-staining was observed in compacted, late 8-cell stage at 64 h post-ovulation as shown in Fig. 3E. At the blastocyst stage (Fig. 3F), positive signals were detected in all cells of the inner cell mass and trophectoderm.

The expression of hsc74 was also examined in embryos fertilized and cultured *in vitro*. As summarized in Table 1, the expression pattern of hsc74 during the time course of development was similar to the data of embryos produced by natural matings. Therefore, we used this *in vitro* system to obtain a large number of embryos at each distinct stage for further examination of mRNA.

hsc74 mRNA expression

First, we examined whether genetic variation can be distinguished at transcriptional level by using RT-PCR followed by SSCP analysis. As shown in Fig. 4A lanes 1-4, the RT-PCR

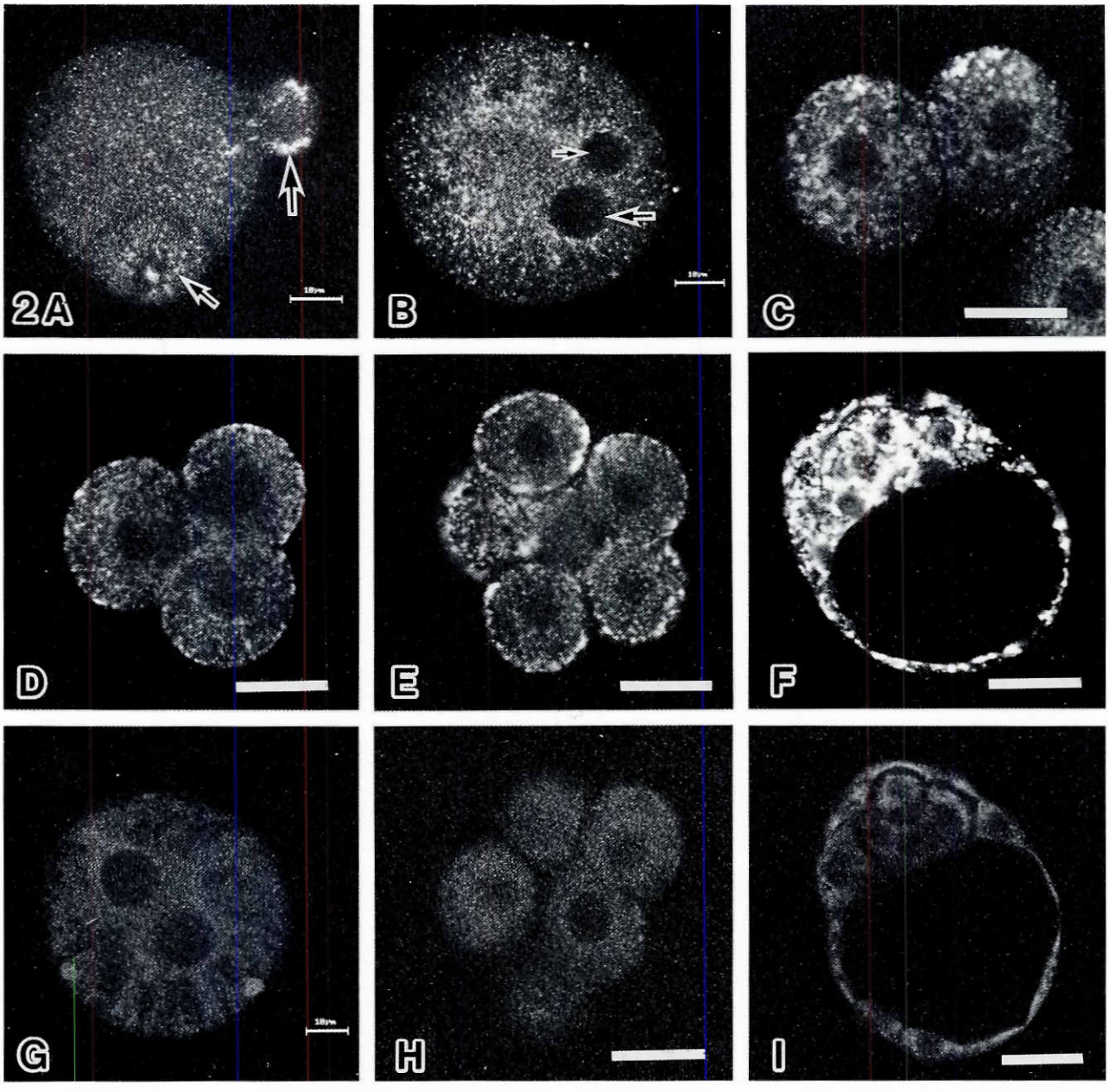

Fig. 2. Confocal images of immunostaining of C3H (A-F) and C57BL/6 (B6) (G-I) embryos with anti-CSA Mab. (A) *One-cell stage C3H embryos at 10 h post-ovulation. Small arrow indicates the presumptive site of sperm head fusion. Strong signals were seen even in the second polar body (large arrow).* **(B)** *One-cell stage at 18 h post-ovulation. Large and small arrows indicate male and female pronuclei, respectively.* **(C)** *2-cell at 34 h post-ovulation,* **(D)** *4-cell at 46 h post-ovulation,* **(E)** *8-cell at 58 h post-ovulation, and* **(F)** *blastocyst at 82 h post-ovulation of C3H. In these stages* **(C-F)**, *the cytoplasm of every blastomere or cell has significant bright signals.* **(G)** *B6 embryos of 1-cell stage at 18 h post-ovulation.* **(H)** *B6 embryos of 8-cell stage at 58 h post-ovulation.* **(I)** *B6 blastocyst at 82 h post-ovulation. B6 embryos in these stages* **(G-I)** *were totally negative with anti-CSA Mab. Bars in A, B, G, 10* μm. *Bars in C, D, E, F, H and I, 25* μm.

products from livers and unfertilized eggs of C3H and B6 could be distinguished after SSCP analysis. In the liver of F1 hybrids between B6 and C3H, both types of transcripts were detected (Fig. 4A, lane 5).

Then, the expression of maternal and paternal hsc74 mRNA during the early stages of the mouse development was investigated in the (B6 X C3H)F1 hybrid embryos made by *in vitro* fertilization. RT-PCR followed by SSCP was carried out using RNAs from unfertilized C3H eggs (13 h post-HCG), unfertilized B6 eggs (13 h post-HCG), 1-cell embryos (12 h post-insemination), 2-cell embryos (24 h post-insemination), 4-cell embryos (48 h post-insemination) and 8-cell embryos (72 h post-insemination). Maternal hsc74^{a} and hsc74^{b} transcripts were constantly detected in both C3H and B6 ovulated eggs, respectively, as well as advanced embryos. The first expression of paternal hsc74^{a} transcripts was detectable in 4-cell stage, 48 h post-insemination.

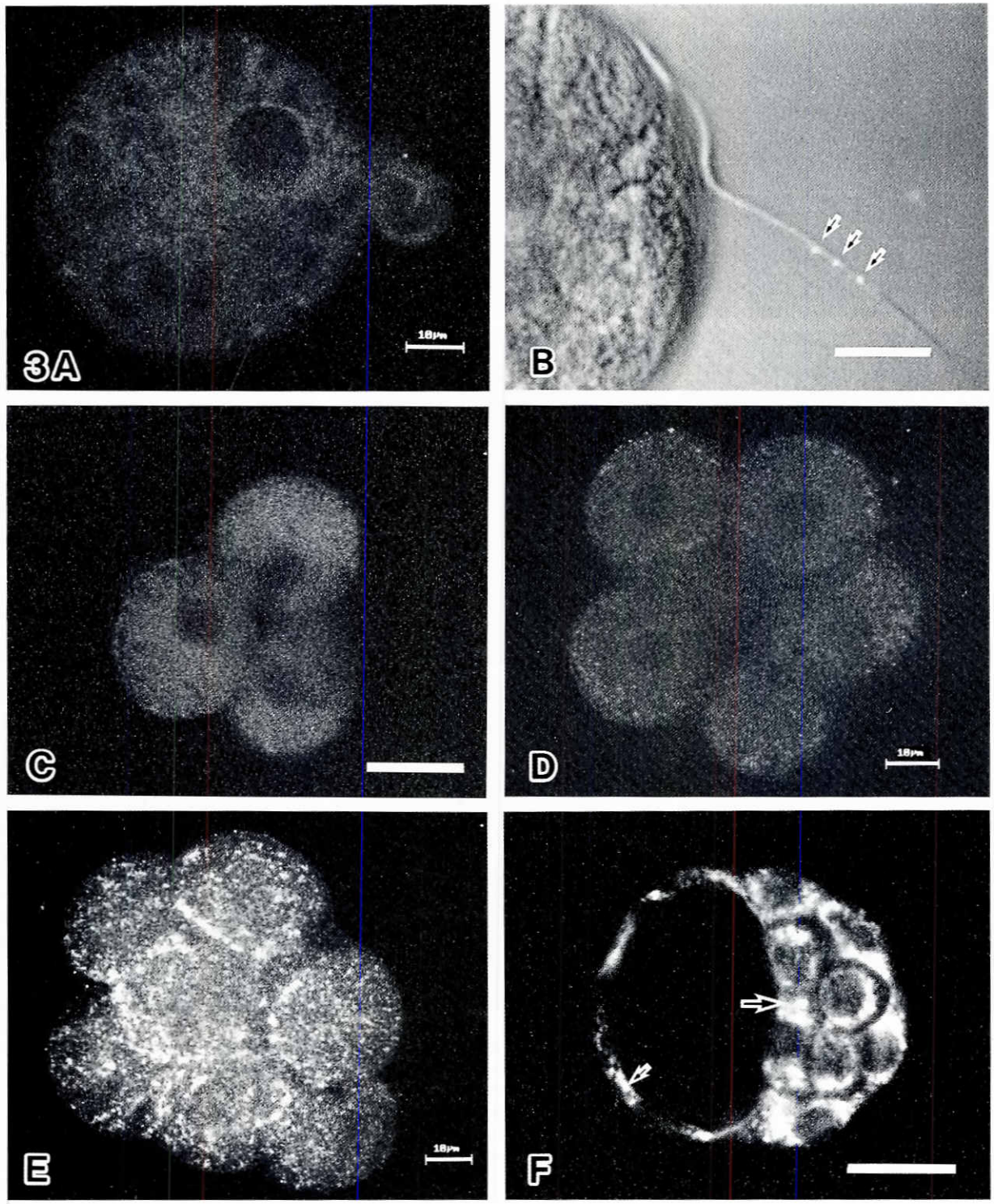

Fig. 3. Confocal images of immunostaining of (B6 X C3H)F1 embryos with anti-CSA Mab. (A) *Pronuclear 1-cell stage at 10 h post-ovulation, positive signals by anti-CSA Mab were not detectable.* **(B)** *B6 eggs fertilized with C3H sperm. In some cases at 10 h post-ovulation, positive granular signals (arrows) were observed in the midpiece of the C3H sperms that were attaching onto the surface of the B6 eggs. Confocal immunofluorescent image was overlaid by transmission image to visualize morphology of the egg and sperm.* **(C)** *4-cell stage at 46 h post-ovulation. No positive signal was detected.* **(D)** *Early 8-cell stage at 58 h post-ovulation. Weakly positive stainings were observed in the peripheral region of blastomeres.* **(E)** *Compacted, late 8-cell stage at 64 h post-ovulation. Bright staining signals were seen in every blastomere.* **(F)** *Blastocyst at 82 h post-ovulation. Positive signals were seen in all cells of the inner cell mass (large arrow) and trophectoderm (small arrow). Bars in A,B,D and E; 10 μm. Bars in C and F; 25 μm.*

The three sets of experiments were performed independently and identical results were obtained. Representative data are shown in Fig. 4B.

Discussion

In many species, the early post-fertilization development of the eggs depends upon the maternal control. In mice, the synthesis of paternally derived β_2-microglobulin is first detectable at the 2-cell stage, indicating embryonic genome activation in this early stage (Sawiki *et al.*, 1981). A recent study about the expression of MHC class I gene in preimplantation mouse embryos demonstrated that mRNA of the H-2D gene was present before the first cleavage division (Sprinks *et al.*, 1993). Some specific proteins synthesized by mouse embryos from the 2-cell to compacted morulae include heat-shock proteins, such as hsp68 and hsp70 (Bensaude *et al.*, 1983). In the present study, we have examined the expression of a novel member of HSP70 family, mitochondrial hsc74, at mRNA and protein level, and demonstrated that the mouse hsc74 is expressed in all preimplantation stages. Moreover, we have established methods to distinguish strain variants both at the protein level using anti-CSA Mab and mRNA level using RT-PCR followed by SSCP analysis. The maternal transcripts and proteins were detected from 1-cell eggs to blastocysts, while the paternal transcripts were detected from 4-cell stage and the paternal protein appeared from 8-cell stage. These data clearly indicate that the activation of embryonic genome for hsc74 occurred as early as 4-cell stage in mice. This study has also confirmed that anti-CSA Mab can be used as a strain specific, intrinsic cell marker from very early developmental stages.

Subcellular localization of the CSA (hsc74) has been demonstrated immunocytologically in mitochondria (Michikawa *et al.*, 1993a). In the present study, we have observed a perinuclear accumulation of CSA-positive signals in the C3H eggs at premetaphase I stage (data not shown). This characteristic distribution pattern is consistent with that of mitochondria at this stage of mouse oocytes, as described previously (Van Blerkom and Runner, 1984; Van Blerkom, 1991). As shown in Fig. 2E, hsc74 protein is localized predominantly in the peripheral region of the blastomeres, but also exists in the perinuclear region, coinciding with the distribution pattern of mitochondria at this stage. We have also immunostained (C3H X B6)F1 embryos derived from C3H females mated with B6 males, and observed the similar staining pattern to that in C3H embryos through the preimplantation stages (data not shown). In contrast, (B6 X C3H)F1 embryos at early 8-cell stage (Fig. 3D) had positive signals only in the peripheral region of the blastomeres. From these observations, the newly synthesized hsc74 protein might be transported specifically into mitochondria in the peripheral region of the blastomeres in the early 8-cell stage embryos.

The anti-CSA antibody was originally established as a mouse monospecific polyclonal antibody against C3H strain specific antigens for the identification of the cell origin in experimental chimeric tissues or tissue recombinant experiments (Kusakabe *et al.*, 1988). Later, we succeeded in obtaining mouse monoclonal antibody against CSA (anti-CSA Mab). The strain specificity of this anti-CSA Mab has been clearly demonstrated by SDS-PAGE and Western blotting (Fig. 1). The cDNA of this antigen has been cloned from cDNA library of C3H kidney by the anti-CSA Mab (Michikawa *et al.*, 1993a). The sequence analysis revealed that this antigen molecule is identical to the peptide-binding protein 74 (PBP74, Domanico *et al.*, 1993) or mortalin (Wadhwa *et al.*, 1993a), a novel member of the HSP70 family. These molecules have been discovered by different ways. Unfortunately, the functions of these molecules are little known so far. The PBP74 was reported to be localized in cytoplasmic vesicles, and possibly not in the nucleus, mitochondria, and plasma membrane (Van Buskirk *et al.*, 1991). Mortalin, on the other hand, was reported as cell mortality marker (Wadhwa *et al.*, 1993a), and may control the cellular mortal and immortal phenotypes with two distinct intracellular distributions, the cytosolic and perinuclear mortalin (Wadhwa *et al.*, 1993b). As reported previously (Michikawa *et al.*, 1993b), there were at least two mRNA species of hsc74 in total cellular RNA. Considering that cDNA sequences of hsc74 (CSA), PBP74 and mortalin are essentially identical (Domanico *et al.*, 1993; Michikawa *et al.*, 1993b; Wadhwa *et al.*, 1993a), there might be different mature forms of the hsc74/PBP74/mortalin gene product .

In conclusion, hsc74 is one of the ubiquitous, house-keeping proteins expressed from very early stages of embryogenesis. Transcripts and proteins of hsc74 are derived only from the maternal gene from 1-cell to 4-cell stages. The paternal mRNA expression begins from 4-cell stage, and the significant increase of the paternal protein occurred from late 8-cell stage.

TABLE 1

EXPRESSION OF PATERNALLY DERIVED hsc74[a] IN (B6 X C3H)F1 EMBRYOS FERTILIZED *IN VITRO*[#]

Time after insemination (h)	No. of embryos (CSA positive/total observed) 1-cell*	2-cell	4-cell	8-cell	16-cell	blastocyst
6	0/32					
15	0/44					
23		0/92				
48	0/6	0/66				
60			0/24	0/8		
72			2/2	54/54	13/13	
96						26/26

[#]C57BL/6 eggs were fertilized by C3H sperm *in vitro*. Embryos were stained with monoclonal anti-CSA antibody. *Only fertilized eggs with two visible pronuclei were evaluated in this stage.

Materials and Methods

Mice

C3H/HeN(C3H) and C57BL/6N(B6) mice were originally purchased from Charles River Japan and Clea Japan, respectively, and maintained in the animal facility of Life Science Tsukuba Research Center at the Institute of Physical and Chemical Research (RIKEN).

Strain specificity of anti-CSA antibody

The strain specificity of the antibody was examined by Western blot analysis of liver extracts from C3H and B6, and by fibroblast culture from day 10 embryos of the two strains according to the method in the previous paper (Kusakabe *et al.*, 1988).

The relative molecular mass of hsc74 was determined as follows. Splenocytes were squeezed out from the spleen by bent needles, and a lymphocyte fraction was separated by Ficoll-Paque (Pharmacia). Depletion

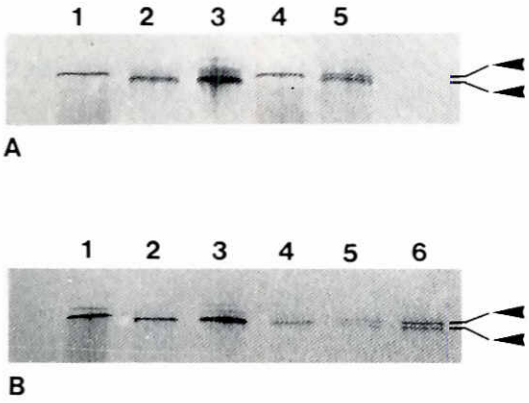

Fig. 4. Expression of genetic variants of hsc74 mRNA detected by SSCP analysis on the RT-PCR products. (A) *Lane 1, C3H liver; lane 2, B6 liver; lane 3, unfertilized B6 eggs; lane 4, unfertilized C3H eggs; and lane 5, (B6 X C3H)F1 liver.* **(B)** *Samples from (B6 X C3H)F1 hybrid embryos made by in vitro fertilization. Lane 1, unfertilized C3H eggs (13 h post-HCG); lane 2, unfertilized B6 eggs (13 h post-HCG); lane 3, 1-cell F1 embryos (12 h post-insemination); lane 4, 2-cell F1 embryos (24 h post-insemination); lane 5, 4-cell F1 embryos (48 h post-insemination), and lane 6, 8-cell F1 embryos (72 h post-insemination). The upper bands indicate the presence of paternal hsc74^a mRNA, and the lower bands indicate the presence of maternal hsc74^b mRNA. The onset of the paternal transcripts was detectable in 4-cell stage. Band-shifts are indicated by arrowheads. Samples for SSCP analysis were prepared from 138 bp RT-PCR products for exon 16 of hsc74 gene (see Materials and Methods).*

of red blood cells was performed by hypotonic shock. Lymphocytes were lysed in 50 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1% Nonidet P-40 on ice for 30 min, sonicated briefly and cell debris was then removed by centrifugation (15000xg). SDS-PAGE was carried out on 10% polyacrylamide gels according to the method of Laemmli (1970). Separated proteins were transferred onto PVDF membrane, preincubated with 5% normal goat serum (NGS), 1% bovine serum albumin (BSA) in PBS, and incubated in anti-CSA Mab. The primary antibody was detected using goat anti-mouse IgG antibody (Zymed) followed by incubation with a mouse monoclonal PAP (Zymed). Bands were then visualized by using a mixture of 3,3'-diaminobenzidine tetrahydrochloride, 0.05 % H_2O_2 and $NiCl_2$.

Embryo collection, in vitro fertilization and culture

Naturally fertilized embryos were collected from non-hormone treated 8-10 week-old females. Embryos of three different genotypes were used; C3H, B6 and B6 X C3H. Embryos were collected 10, 18, 34, 46, 58, 64 and 82 h post-ovulation according to procedures described by Hogan *et al.* (1986). Predominant stages of embryos at 10, 18, 34, 46, 58, 64 and 84 h post-ovulation were 1-cell (fertilization, second polar body extrusion to pronuclear stages), 1-cell (pronuclear stage with both female and male pronuclei), 2-cell, 4-cell, 8-cell, compacted 8-cell and morula/blastocyst stages, respectively. Apparently degenerating or retarded embryos were not used in this experiment. Superovulation was also employed to produce *in vitro* fertilized embryos (Hogan *et al.*, 1986). The F1 embryos were produced by inseminating B6 eggs with C3H sperm. Control embryos were produced by *in vitro* fertilization of B6 eggs by B6 sperm (as a negative control) or C3H eggs by C3H sperm (as positive control).

Whole-mount immunocytochemistry of embryos

The zona pellucida was removed by incubation with 0.5% (w/v) pronase (Boehringer Mannheim) dissolved in M2 medium, followed by two rinses in M2 before fixation. The zona-free embryos were transferred into ice cold 95% ethanol containing 1% acetic acid with minimal volume of the M2 by a mouth-controlled glass micropipette. For optimal preservation of the hsc74^a antigenicity, embryos were fixed for 1 h. Subsequently, the embryos were dehydrated in ice cold 100% ethanol, rehydrated slowly with 90, 80, 70, 60 and 50% ethanol series, and rinsed with distilled water. For immunostaining, embryos were preincubated for 1 h in blocking solution, PBS(-) containing 5% NGS and 1% BSA, and incubated overnight with anti-CSA Mab at 1:100 in the blocking solution. After efficient rinse with PBS(-), embryos were incubated for 1 h in 1:50 dilution of goat anti-mouse IgG(H+L)-FITC-conjugated second antibody (Zymed). After four washes in PBS(-), the embryos were transferred to a small drop of the mounting fluid (Difco) on a glass slide. A spacer made of a piece of laboratory film (Parafilm, American National Can, USA) with a hole in the center was inserted between a glass slide and a cover glass to avoid compression of the embryos. The whole-mount specimens were observed under the LSM 10 confocal laser microscope (Carl Zeiss, Germany). In each experimental group, a minimum of 10 eggs or embryos were examined.

RNA preparation

Total RNA was extracted from eggs, *in vitro* fertilized embryos (80 of each), and liver of adult mice (as control RNA) by a standard single-step guanidium isothiocyanate method (Chirgwin *et al.*, 1979). The eggs and embryos were pretreated with pronase solution as described above to remove cumulus cells and the zona pellucidae. The final RNA concentration was determined by spectrophotometric method. To remove the contaminating DNA, incubation with RNase free DNase I (Boehringer Mannheim) was used before following assays.

Reverse transcription-polymerase chain reaction and single-strand conformation polymorphism analysis

About 500 ng of total RNA was reverse transcribed and amplified by the following method. One µl oligo $(dT)_{12\text{-}18}$ (500 µg/ml) primer was added to 12 µl DNase I treated RNA sample and the mixture was then incubated for 10 min at 70°C, followed by rapid chilling on ice. Four µl of 5x concentrated first strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), 2 µl of 100 mM DTT, 1 µl of mixed dNTP stock (10 mM of each dATP, dGTP, dCTP and dTTP) was then added to the RNA sample. The mixture was overlaid by light mineral oil (Sigma) and placed at 37°C for 2 min to equilibrate the temperature. 1 µl of RNase H- reverse transcriptase Superscript II (Gibco BRL) was added and reverse transcription was performed by incubation for 1 h at 50°C. Following reverse transcription (RT), the RT product was heated at 99°C for 5 min.

DNA amplification was carried out in 100 µl containing 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 µM each primer and 2.5 units of Taq polymerase (Boehringer Mannheim), and performed on thermal sequencer (Iwaki) after an initial denaturation step (94°C for 240 sec). The repetitive cycle was as follows: denaturation for 85 sec at 94°C, annealing for 150 sec at 60°C, and extension for 180 sec at 72°C (35 cycles). The oligonucleotide primers (5' - GCA ACA AGC TAA AGG AAG AGA TTT CC - 3' and 5' - TTT TGT ACG CCA TTT CGA AGA GTT TC - 3') were based on mouse cDNA sequence (Michikawa *et al.*, 1993a) and the region in exon 16 of the *hsc74* gene amplified to generate a 138 bp fragment. This region contains a single base transition of G in C3H to C in B6. 10 µl of each of the PCR products were electrophoresed in 2% agarose gel followed by ethidium bromide staining. If further amplification was required, a sample of the amplified DNA was diluted (20-50x) and used as the template for the second round of PCR. Finally, the amplified fragments were recovered and purified from gel cast with low-melting-temperature agarose (FMC) by a standard method (Maniatis *et al.*, 1982).

In order to determine the time when paternal hsc74 transcript first appears during the development, RT PCR-SSCP analysis (Mohabeer *et al.*, 1991) was used. Briefly, 2 µl of the PCR products was mixed with 2 µl of formamide, heated to 85°C for 4 min and chilled quickly. Polyacrylamide gel electrophoresis was carried out on PhastGel media (8-25 % pre-made

gels) using PhastSystem (Pharmacia) and silver stain (PhastGel silver kit) according to the manufacturer's manual to visualize single-strand DNA.

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