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Identification and Characterization of a Constitutive HSP75 in Sea Urchin Embryos

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An antiserum against a hsp of the 70-kDa family was prepared, by means of a fusion protein, which was able to detect a constitutive 75-kDa hsc in the sea urchin *P. lividus*. This hsc was present both during oogenesis and at all developmental stages. A two-dimensional electrophoresis has revealed four isolectric forms of this 75-kDa hsc. The amino acid sequence of the fragment used to prepare the anti-hsp70 antibodies revealed a 43% identity with the corresponding part of sea urchin sperm receptor, and in mature eggs a brighter immunofluorescence was seen all around the cell cortex where the receptor for sea urchin sperm is localized. In oocytes the hsp75 was localized in the cytoplasms but not in the nuclei. In the embryos a higher hsp75 concentration was found in the portion facing the lumen of the cells which invaginate at gastrulation.

In all prokaryotic and eukaryotic cells when the basal temperature rises, a special set of proteins referred to as heat shock proteins or stress proteins are immediately synthesized. These proteins counteract heat damage which would otherwise cause denaturation and aggregation in misfolded conformation of other cell proteins (1-5). This mechanism also protects cells against and facilitates recovery from a wide variety of environmental stress conditions (6,7). The heat shock proteins of the 70kDa family are the most conserved phylogenetically (8-11). Some forms are only heat-inducible (hsp70) but others are constitutively expressed, although in lower amounts, in non-stressed conditions (hsc70). The basal functions of these have emerged from studies which showed them to be involved in binding other polypeptides to prevent their precocious folding untill final intracellular localization is reached (12-20). Hsc70 expression is also observed in embryonic development (21). *Paracentrotus lividus* embryos belong to a Mediterranean sea urchin species, which is well known as a developmental model system. In *P.lividus* embryos the shift of temperature from 20°C to 31°C elicits hsp70 mRNA synthesis in all developmental stages (22). Previous work has provided information about hsp70 synthesis and function under heat shock (23,24). On the contrary it was difficult to study and to obtain information about *P.lividus* constitutive hsc70, because of its low level of synthesis. In fact in *P.lividus* embryos the constitutive hsc70 mRNA is basally expressed at very low level (25). Therefore a hsp70 sea urchin fusion-protein was prepared, and antibodies against it obtained. These were used to study the presence of hsc70 both in oogenesis and during the stages of development.

MATERIALS AND METHODS

Embryo culture and labeling of the embryos. *Paracentrotus lividus* embryos were reared at 10,000/ml in millipore filtered sea water, at the physiological temperature of 20°C, from the fertilization on.

Heat shock embryos and in vivo protein labeling. Embryos at gastrula stage were subjected to heat shock at the temperature of 31°C for 45 min. and 35S-methionine, 1000 Ci/mM (Amersham) was added 30 min. before harvesting, to the final concentration of 50 μCi/ml.

Construction of recombinant expression pEx31 vector. The 5′ NcoPstI restriction fragment of the 3321Nclone (25) filled-in in Nco site was subcloned into the BamHI (filled-in)-PstI sites of each frame of pEx31 expression vector (26) using the usual procedure (27). *E.coli* K12 H strain was used for transfection. Recombinant clones were selected for the presence of 24 kDa MS2 fusion-protein (26,28) on SDS-PAGE (29) followed by Western-blot procedure (30) using anti-MS2 monoclonal antibodies to test the specificity of the fusion-protein. pEx31 plasmids and anti-MS2 monoclonal antibodies were a kind gift of Dr. M. Di Carlo.

Preparation of anti-hsp70 immune serum. The 24 kDa fusion-protein was cut from the gels, electroeluted by an electro-eluter apparatus (Bio-Rad) and aliquots (50μg of protein in incomplete Freund's adjuvant) were weekly injected in two New Zealand White rabbits for 5 weeks. Anti-hsp70 serum was stored at minus 20°C.

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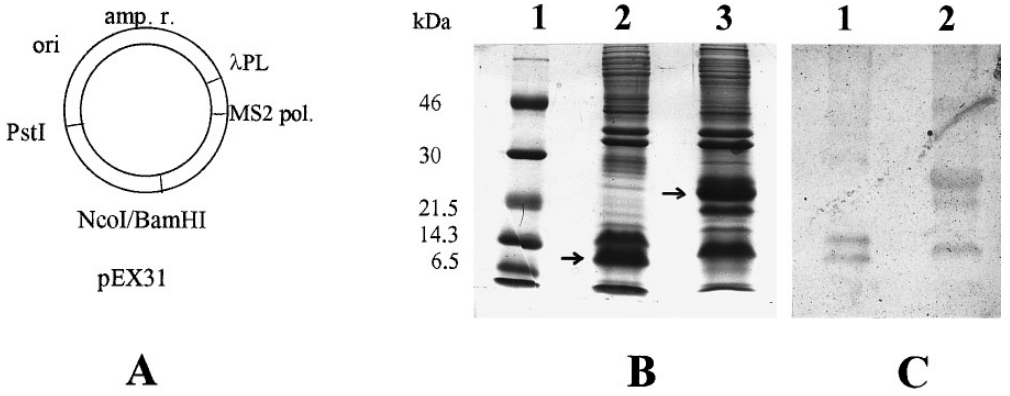

FIG. 1. (A) Scheme of the construction recombinant expression plasmid pEx31. The DNA fragment of *P. lividus* hsp70 gene IV (25) of 367 bp from restriction sites NcoI-PstI was subcloned into BamHI-PstI sites of pEx31 polylinker just beyond the MS2 fage polymerase gene. Nco and BamHI restriction sites were filled-in to blunt ends. Abbreviations: λPL, the lambda PL promoter; MS2 pol., DNA of the MS2 polymerase subunit; amp.r., ampicillin resistance; ori, origin of replication. (B) Expression of the fusion-protein. MS2 polymerase protein subunit fused to the most conserved part of hsp70 observed on SDS-PAGE after staining with Coomassie blue. Lane 1, the electrophoretic mobility of the standard proteins; lane 2, arrow indicates the 12 kDa MS2 polymerase subunit overexpressed by the pEx31 plasmid in the total proteic lysate of bacteria; lane 3, arrow indicates the 24 kDa fusion-protein overexpressed by recombinant plasmid pEx31 in the total proteic lysate of bacteria. (C) Identitfication of 24 kDa fusion-protein. Immunoblot of the samples of lanes 2 and 3 of B reacted with anti-MS2 polymerase monoclonal antibodies, lanes 1 and 2 respectively.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was carried out according to O'Farrell (31). Samples of 400 μg total proteic lysate of non heat-stressed and non labeled gastrula embryos was loaded on first-dimension in 1.6% pH5-7 and 0.4% pH 3-10 ampholytes (LKB) and run overnight at 300V. To determine protein isoelectric points, the pI calibration kit from Pharmacia Biotech was used. Western blot procedure (30) followed the second SDS-PAGE dimension was run at 100V for 90 minutes. The anti-hsp70 antibodies specific to *P.lividus* were used at a 1:500 dilution and second antibodies IgG AP conjugate (Amersham), were then added; ECL Hybond filter (Amersham) were used.

Immunoprecipitation reactions. These were performed adding preimmune rabbit serum or anti-hsp70 rabbit serum (10 μl) to total proteic lysate of heat-treated gastrula embryos 'in vivo' labeled with 35S-methionine and incubating overnight at 4°C. The total proteic lysate was obtained by sonication in 1mM EDTA, 1% Triton with the addition of 1mM PMSF. The samples were subjected to 10% SDS PAGE. Gels were soaked with 2,5-diphenyloxazole in dimethyl sulfoxide solution and exposed for autoradiography to Kodak RP X-Omat films.

Immunoistochemistry. Ovaries and embryos at the desired stages were fixed with Bouin fixative for 2 h at room temperature. After gradual dehydration in ethanol they were included in paraffin at 56°C. Sections (5 μm thick) were picked up on clean glass slides, deparaffinated, and rehydrated by PBS (phosphate buffered saline) and allowed to dry in air. Anti-hsp70 antibodies in PBS containing 5% horse serum (Amersham), 0.02% NaN3 were added onto slides for 16 h in a humidified chamber at room temperature. After washing

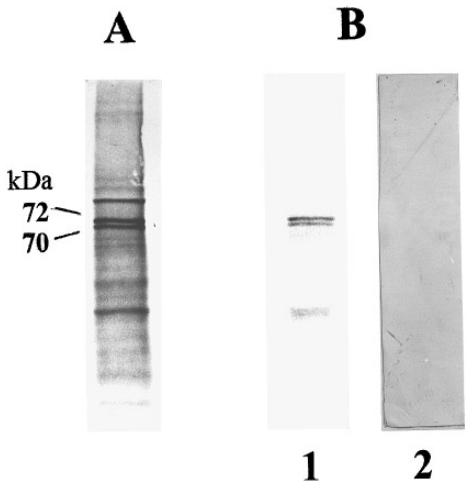

FIG. 2. Immunoprecipitation of hsp72 and hsp70. (A) Electrofluorogram of the bulk proteic lysate of heat-treated gastrula embryos *in vivo* labeled with ^{35}S-methionine. (B) Electrofluorogram of the immunoprecipitate from the total proteic lysate obtained as in A, using immune serum containing polyclonal antibodies against hsp70 fusion-protein, lane 1, or preimmune serum, lane 2.

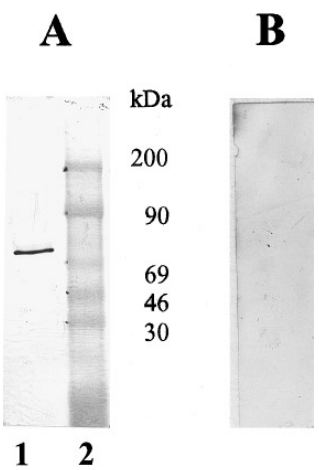

FIG. 3. Identification of the hsc75. (A) Lane 1, immunoblot of total proteic lysate of non-stressed gastrula embryos reacted with the anti-hsp70 immune serum; lane 2, standard markers. (B) Immunoblot as in A using preimmune serum.

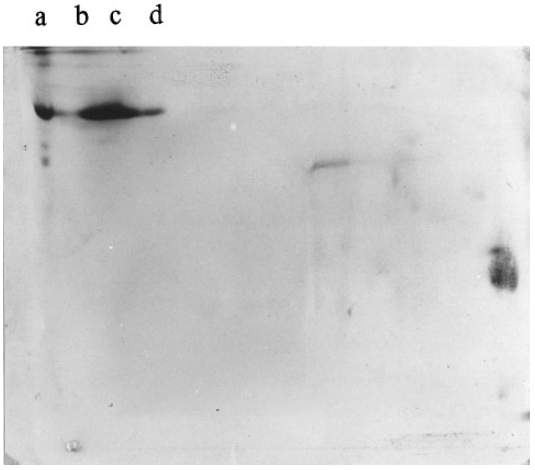

FIG. 4. Two-dimensional electrophoresis of total proteic lysate of non heat-stressed gastrula embryos followed by immunoblot after incubation with anti-hsp70 immune serum. a-d indicate the pI, calculated by markers in a parallel gel. a, 8.8 pI; b, 8.2 pI; c, 7.9 pI; d, 7.6 pI.

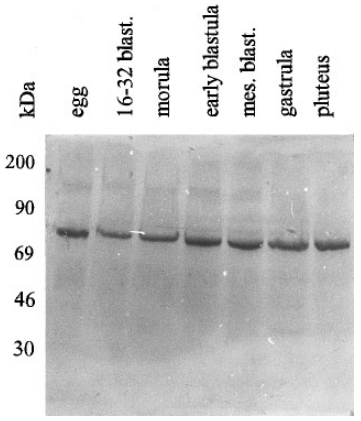

FIG. 6. Immunoblot of total proteic lysate of non-stressed embryos at different developmental stages reacted with anti-hsp70 immune serum. Lane kDa, standard marker molecular weights. The developmental stages are indicated above. Abbreviations: 16-32 blast., 16-32 blastomeres; mes. blast., mesenchyme blastula.

a second fluorescent antibody was added (Amersham) and incubated for 2 h. Photographs were obtained at Zeiss IM inverted fluorescence microscope.

RESULTS AND DISCUSSION

Antibodies anti-hsp70 by a fusion-protein and their specificity. As mentioned above hsc70 is only present in very low amounts so, we synthesized a part of it as a fusion-protein in *E.coli* to be used as an antigen in rabbits. In order to obtain this fusion-protein the DNA fragment from 1979 bp to 2247 bp of the *P.lividus* hsp70 gene IV (25) codifying for the most highly conserved part among the hsp70 in different species, was subcloned in the pEX 31 expression vector following the MS2 DNA polymerase subunit gene (fig. 1A). The molecular weight of the resulting fusion-protein expressed in *E.coli* was of 24 kDa, as expected (fig. 1B, lane 3) and the anti-MS2 polymerase monoclonal antibodies recognized the 24 kDa fusion-protein by immunoblot (fig.1C, lane 2). An immune serum was then raised in rabbits by injection of 24 kDa protein-fusion. Its specificity against *P.lividus* hsp70 family was ascertained by immunoprecipitation of the two known hsps of 72 and 70 kDa from total radiolabeled proteic lysate of the embryos subjected to heat shock. These two hsps are known to be synthesized exclusively under heat shock as previously described (23,24) and are detectable only if they are radiolabeled *in vivo* as shown by

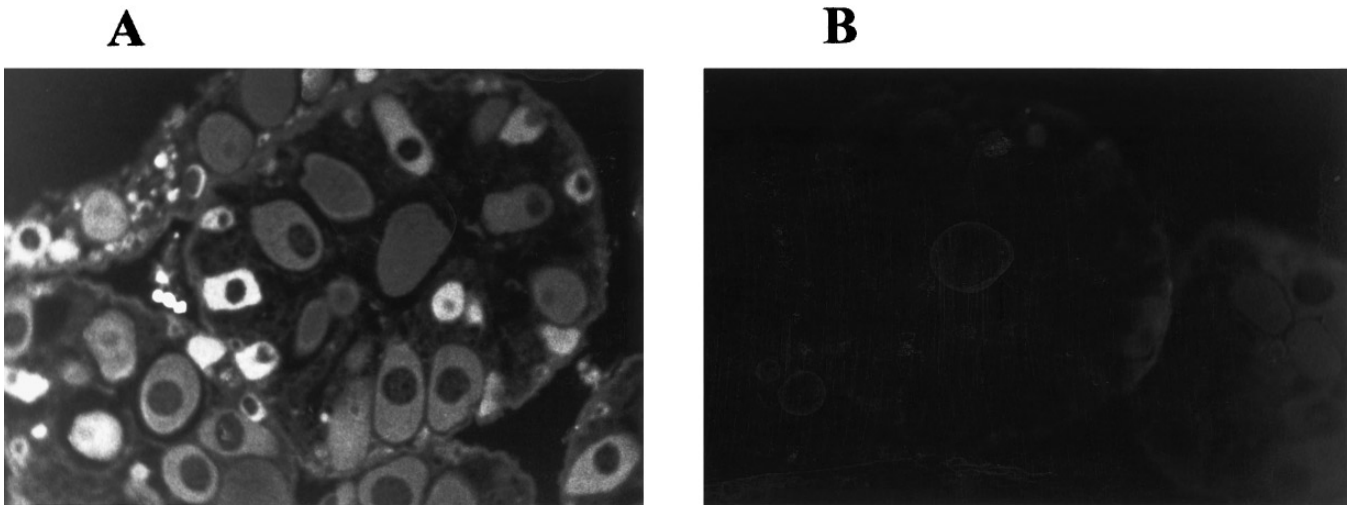

FIG. 5. Immunofluorescence staining of hsc75 in ovary. (A) Section of ovary subjected to immunofluorescence staining after anti-hsp70 immune serum reaction. (B) Section of ovary subjected to immunofluorescence staining after preimmune serum reaction.

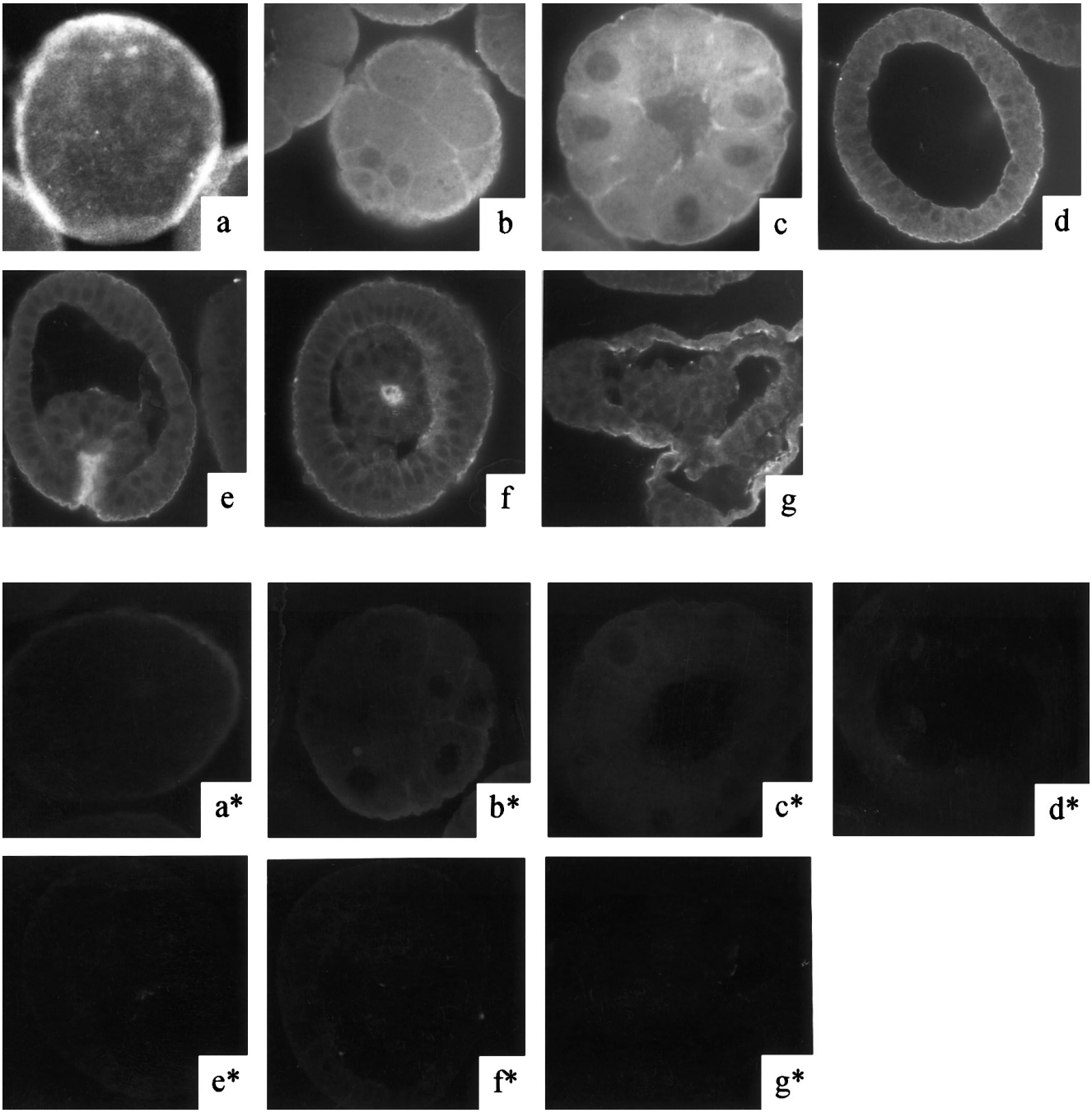

FIG. 7. Immunofluorescence staining of hsc75 in embryos. Section of unfertilized eggs (a) or embryos at different developmental stages: (b) 32 cells, (c) morula, (d) blastula, (e) gastrula sectioned along the animal-vegetal axis, (f) gastrula sectioned through the animal-vegetal axis along a subequatorial plane, and (g) pluteus, subjected to immunofluorescence staining after anti-hsp70 immune serum reactions. a*-g* sections of the same developmental stages subjected to immunofluorescence staining after preimmune serum reactions.

the electrofluorogram of fig. 2A. In fact, because of the low amount present they are not detectable with chemical methods. The antibodies of the immune serum are able to recognize and to precipitate only the two known hsp70 from the total radiolabeled proteins, which indicates that they are specific for the hsp70 family members. This was also ascertained by fluorography following the SDS-PAGE separation of the immunoprecipitates (fig. 2B, lane 1). The preimmune serum, used as control, is not able to precipitate either the hsp70 or other proteins (fig. 2B, lane 2).

Detection of P. lividus constitutive hsc75 and its characterization. The immune serum was then used to detect the constitutive member of hsp70 family by immu-

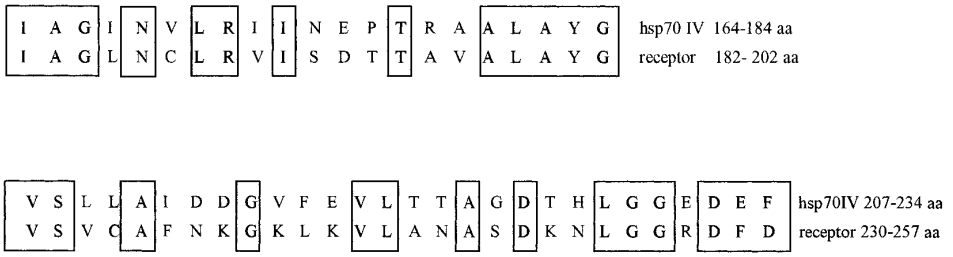

FIG. 8. Comparison of aa sequence coded sperm receptor gene (32) with that coded by the hsp70 gene IV (25). The amino acid identities are boxed.

noblot analysis. Total proteins from non heat-treated and non radiolabeled embryos were separated on SDS-PAGE, blotted and reacted with anti-hsp70 immune serum. The results show a single component (fig 3A, lane 1), with a molecular mass of 75 kDa, which is somewhat higher than that of the known hsp72 and hsp70 synthesized under heat-shock. So all members of the *P.lividus* hsp70 family detected up to now have slightly different molecular weights, the constitutive one being the largest. The preimmune serum does not show any reaction (fig.3B). The hsc75 which appears as a band on SDS-PAGE was further characterized by immunoblot analysis of a two-dimensional electrophoresis gel. The analysis indicates that the hsc75 has four isoforms all localized in the basic range (fig. 4). The most basic variant has a pI of 8.8 (a in fig.4), another two isoforms are very close (b,c) and their p.I. are 8.2 and 7.9 respectively; finally there is an isoform (d) at 7.6 pI which is expressed at a very low level.

hsc75 in oogenesis. In order to investigate about the presence of the constitutive hsc75 during oogenesis, oocytes of different sizes were observed in sections of ovaries using immunofluorescence staining with anti-hsp70 immune serum. The analyses revealed that all oocytes contained the hsc75 in their cytoplasms but not in the nuclei (fig. 5). The medium and the large oocytes seemed to be less strongly immunostained than the small ones. This might also be due at least in part to a higher dispersion of the fluorescence in a larger cytoplasm.

hsp75 during the development. The presence of hsc75 has been analyzed in embryos of several developmental stages by immunoblot analysis first and then by immunofluorescence staining. As shown in fig. 6, hsc75 is found in the total proteic lysate of embryos at all stages analyzed. Immunofluorescence staining was observed on sections of unfertilized eggs and embryos. In unfertilized eggs a widespread hsc75 immunofluorescence was found with a reinforce at the egg periphery. The presence of hsc70 in unfertilized eggs may indicate that the hsc70 synthesized during oogenesis is preserved in the mature eggs (fig.7a). What is the meaning of the brighter immunofluorescence seen all around the cell cortex? The receptor for sea urchin sperm has been shown to have some amino acid sequence homology with the hsp70 (32). The aminoacid identity calculated on the hsp70 fragment used to prepare the fusion-protein and on the corresponding part of receptor for sea urchin sperm was 44%. Two parts are especially highly conserved (fig.8). One has 61.9% and the second has 50% identity. The brighter immunofluorescence of the egg cell surface may therefore depend on the presence of the sperm receptor which cross-reacts with the anti-hsp70 immune serum or on the presence of the hsc75 close to the cell surface. In 32 cell stage embryos (b, fig. 7) and in all following stages of development, such as morula (c), blastula (d), early gastrula (e), gastrula (f) and pluteus (g), the hsc75 was found in all cells, confirmig its fundamental role also during development. The immunofluorescence staining was found in the cytoplasm while in the nucleus it was not significant. Looking at the invaginating portion at the beginning of gastrulation in sections taken along the animal-vegetal axis (e, fig. 7) or across it along a subequatorial plane (f) a more intense staining was found on the surface of the invaginated cells facing the lumen. An explanation of this may be that the hsc75 are involved in this specific side of those cells which are building a new three dimensional structure and are transiently changing shape. This is reasonable taking into account the role of hsc75 members as chaperones. It is possible that in these cells the basal level of hsc75 synthesis is higher. In previous work carried out by in situ hybridization of heat-treated embryos we have detected a lower hsp70 mRNA synthesis in the intestine cells than the ectodermic cells (33). Also, the hsp72 and hsp70 synthesis appears higher in isolated ectodermic tissues than in endodermic ones of the heat-treated embryos (34). The lower heat shock response in these latter ones might be due to the higher basal presence of hsc75.

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