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Original Article

Does the chaperone heat shock protein hsp70 play a role in the control of developmental processes?

NICOLE ANGELIER*, NICOLE MOREAU, MARÍA-LUZ RODRIGUEZ-MARTIN, MAY PENRAD-MOBAYED and CHRISTELLE PRUDHOMME

Laboratoire de Biologie Moléculaire et Cellulaire du Développement, UA1135 CNRS-Université Pierre et Marie Curie, Paris, France

ABSTRACT **Expression of an *hsp70* gene strictly inducible in somatic cells and constitutively expressed during oogenesis was investigated during embryogenesis of the amphibian *Pleurodeles waltl*. Results from Northern hybridization experiments and RNase protection assays provided evidence for the presence of inducible hsp70 mRNA under normal conditions at every embryonic stage. Immunoblotting of embryo proteins separated by 2D-electrophoresis provided evidence for the presence of a single polypeptide of about 74 kDa likely to be an HSP70-related protein, from unfertilized egg to tailbud stage. Immunocytological analysis showed that HSP70-related proteins were localized in the cytoplasm of all blastomeres. It also pointed out that nuclear transfer of the protein occurs in certain cells, precisely at the time of their invagination and subsequent internalization during normal *Pleurodeles* development. Such nuclear transfer involves involuting mesodermal cells in the blastopore region at the time of gastrulation. It also involves neurodermic cells at the time of neural tube closure. Interestingly, in exogastrulas nuclear transfer did not occur in cells which could no longer invaginate. Such behavior of HSP70-related proteins led us to suggest that they are involved in the control of nuclear activity associated with important developmental events such as cellular internalization processes. Such a role may be a direct consequence of HSP70-related protein functional properties as molecular chaperones.**

KEY WORDS: *HSP70, molecular chaperones, developmental events*

Introduction

Temperature upshift and other types of environmental stress induce the synthesis of a small set of evolutionarily-conserved proteins called heat-shock proteins (HSPs). This protective response to stress is almost universal, occurring in prokaryotes as in eukaryotes. The 70 kDa heat-shock protein (HSP70) has proven to be the most highly conserved, from bacteria to human, and belongs to a protein family that includes forms expressed under optimal growth conditions (constitutively-expressed forms, heat-shock cognate, HSC70) and stress-inducible forms (HSP70). They have been reported in a wide variety of eukaryotic non-stressed cells from yeast, *Drosophila*, amphibians, mice and humans (Bienz and Gurdon, 1982; Bensaüde *et al.*, 1983; Craig *et al.*, 1983; Zimmerman *et al.*, 1983; Kurtz *et al.*, 1986; Palter *et al.*, 1986; Wu and Morimoto, 1986; Heikkila *et al.*, 1987; Rosario *et al.*, 1992). The corresponding genes have been cloned and sequenced in many species (for review, see Pelham, 1982; Lindquist and Craig, 1988; Craig and Gross, 1991).

Proteins of the HSP70 family are "molecular chaperones" which may interact with other proteins under normal environmental conditions as well as under stress, thus preventing the aggregation of nascent or unfolded proteins, facilitating inter- and intramolecular interactions, and improving protein transfer through the membranes of cellular organelles (for review, see Ellis and Van der Vries, 1991; Heindrick and Hartl, 1993) and transfer from the cytoplasm to the nucleus (Mandell and Feldherr, 1990).

In terms of amphibian development, such HSP70-related proteins have been identified in *Xenopus laevis* non-stressed oocytes and embryos (Bienz and Gurdon, 1982; Browder *et al.*, 1987; Heikkila *et al.*, 1987; Horrell *et al.*, 1987; Herberts *et al.*, 1993). However, Bienz (1984) reported that in *Xenopus*, the only cells found to contain hsp70 mRNA without heat shock were the oocytes.

Recently, we provided evidence for constitutive expression of a somatic heat-inducible *hsp70* gene during oogenesis in another amphibian, *Pleurodeles waltl* (Billoud *et al.*, 1993). Furthermore, we showed that HSP70-related proteins are progressively transferred to the nucleus in the course of oogenesis (Billoud *et al.*, 1993). It has been suggested that these proteins mediate the nuclear transport of proteins involved in the control of transcriptional processes (Moreau *et al.*, 1994).

We sought to determine whether or not such inducible gene is constitutively expressed during *Pleurodeles* embryogenesis. The present paper focuses on this problem. We provide evi-

***Address for reprints:** UA1135 CNRS-UPMC, Groupe Gènes et Développement, Equipe Angelier, Bât. C 6ème ét. case 16, 9 quai St-Bernard, 75252 Paris Cedex 05, France. FAX: 33.1.44272622. e-mail: gda@ccr.jussieu.fr

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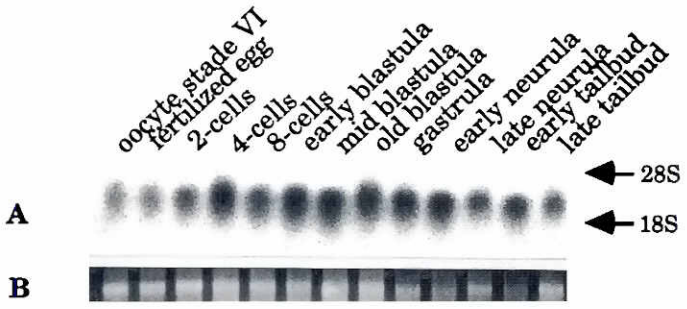

Fig. 1. Northern blot hybridization of stage VI oocyte and embryo stage RNA. *Total RNA of 10 oocytes or embryos from unfertilized egg to tailbud stage was fractionated on agarose gels, stained with ethidium bromide to evaluate loading and RNA integrity, transferred to membranes and hybridized with [^{32}P] Pw70/534 DNA probe.* **(A)** *Signal corresponding to an RNA of about 3kb was detected at every stage. "28S" and "18S" indicate the migration position of rRNA.* **(B)** *As a control of RNA integrity, "28S" rRNA was visualized by ethidium bromide staining.*

dence for the constitutive presence of inducible hsp70 mRNA at every stage of embryogenesis. We also show that HSP70-related proteins are present throughout development. HSP70-related proteins, which are localized in the cytoplasm of all embryonic cells, were found to be transferred to the nucleus of specific cells at defined moments in development. The significance of such specific transfer is discussed in relation to the well known chaperone function of HSP70 proteins.

Results

Developmental expression of hsp70 mRNA

We previously isolated and characterized a full-length cDNA sequence coding for heat shock protein (HSP70) of the amphibian *Pleurodeles waltl*. This gene was found to be constitutively expressed in stage VI oocytes, i.e. under normal conditions (Billoud *et al.*, 1993). In order to determine whether this sequence is constitutively expressed during embryogenesis, we performed Northern blot and RNase protection assays on embryos. We hybridized the least conserved part of this sequence, i.e. the 3' COOH-terminal part called Pw 70/3' (534bp), with Northern blots of RNA from stage VI oocytes, eggs and embryos at every stage of development under normal conditions. A strong signal corresponding to a transcript of about 3kb was observed in RNA from oocytes, eggs and embryos (Fig. 1). However, due to the high homology between the cognate and the inducible forms of *hsp70* genes, we could not exclude the possibility that such a signal visualized either the cognate hsc70 mRNA or the inducible hsp70 mRNA, or both. In order to detect possible constitutive expression during embryogenesis of the inducible hsp70 mRNA, RNase protection assays were performed. An 850 base single-strand antisense cRNA probe was generated from a *Pvu*II digest from the plasmid containing Pw70/3' (534bp). Figure 2 shows that hybridization with RNA from stage VI oocytes under normal conditions led to the protection of about 500 bases of the probe, i.e. almost the same size as Pw 70/3'. Analysis of results in embryos showed strong evidence that inducible hsp70 mRNA is present at every stage of development, from the fertilized egg to the tailbud. An RNA protected fragment of about 500b, i.e. of the same size as that detected in oocytes and positive control, was detected by a significant signal present for each of the various embryogenesis stages (Fig. 2). These results therefore led us to conclude that inducible hsp70 mRNA is constitutively present in embryos.

Developmental expression of HSP70-related proteins

Specificity of the antibody

HSP70 expression was investigated by immunoblots using the monoclonal anti-HSP70 m Ab H_3F_{18} previously characterized by Mattei *et al.* (1989). The 16 amino acid epitope recognized by m Ab H_3F_{18} begins about 160 residues upstream from the C-terminal amino acid of HSP70. This antibody has previously been shown to specifically recognize a protein of $74x10^{-3}$ Mr present in *Pleurodeles* oocytes under normal conditions. In the present study, the specificity of m Ab H_3F_{18} antibody was checked on Western blots from two-dimensional electrophoresis proteins from every stage of embryogenesis. As was the case for oocyte proteins at every embryogenesis stage considered, this antibody specifically recognized only one polypeptide of $74x10^{-3}$ Mr, probably an HSP70-like protein constitutively present in *Pleurodeles* embryos under normal conditions (Fig. 3).

Intracellular localization of HSP70-related proteins in Pleurodeles embryos

Distribution of HSP70-related proteins in *Pleurodeles* embryos was then investigated by immunofluorescence on sections using antibody H_3F_{18}. Throughout embryogenesis, the HSP70 protein was found in the cytoplasm of every blastomere. However, depending on the cells, nuclear transfer of HSP70 occurred in defined moments in embryonic development.

Thus, at the beginning of embryogenesis during the cleavage period, the location of HSP70 protein in nuclear area visualized in late oogenesis was maintained. Such localization is illustrated in Figure 4A,B, taking as examples 2-cell and early blastula stages. To determine whether the HSP70 location is related to the condensed state of chromatin during the cleavage period, we analyzed its location during mitotic phases. It is noteworthy

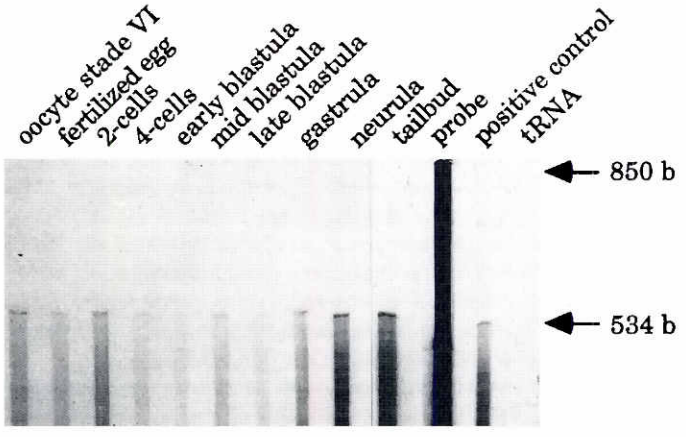

Fig. 2. RNase protection assay of an 850-base ^{32}P-labeled probe consisting of Pw70/534 b+320 b from the vector. *P, probe, positive control with sense Pw70/534 cRNA. RNA from every embryo stage protects the probe from degradation.*

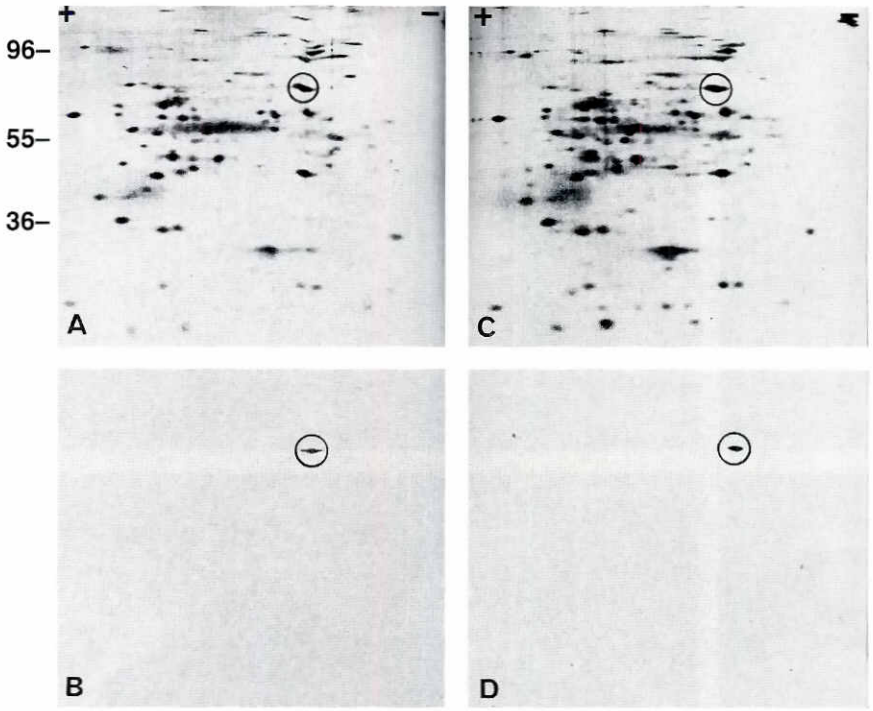

Fig. 3. Specificity of the monoclonal antibody H_3F_{18}. (A-B) *Two-dimensional electrophoresis of a 10,000g supernatant from 2-cell* **(A)** *and gastrula stages* **(B)** *of Pleurodeles waltl. Circles indicate the position of HSP70-related proteins. Silver staining according to Morrissey (1981). On the left, molecular mass markers (Dalton).* **(C,D)** *Corresponding immunoblots after incubation with anti-Plasmodium falciparum HSP70 monoclonal antibody (m Ab H_3F_{18}).*

that in interphase nuclei, HSP70 is associated with the interspersed chromatin. In contrast, HSP70 binds neither to the condensed chromatin of the prophase (Fig. 4E), nor to individualized chromosomes of the metaphase (Fig. 4F) and telophase (not shown). HSP70 is associated only with the fibers of the spindle.

At the beginning of gastrulation, when the blastopore was just forming, the HSP70 protein still exhibited its preferential perinuclear location in the endodermal cells, but no signal was observed in the nuclear regions of external cells or in bottle cells of the blastoporal groove. However, a strong signal was detected in the neck of bottle cells towards the outside of the embryo (Fig. 5A,B). As gastrulation progressed, the HSP70 location remained the same as in the cleavage stage, except for cells of the involuting and involuted dorsal mesoderm (Fig. 5C,D). In those cells, a strong nuclear signal was observed, visualizing the nuclear transfer of the protein. Such transfer was never detected in cells of the external area. As gastrulation reached completion and the yolk plug was formed, the same nuclear transfer of HSP70 was observed in involuted cells of the dorsal and ventral mesoderm, whereas such transfer did not occur in ectodermal cells (Fig. 5E,F). It is noteworthy that HSP70 was located in all the cellular membranes. To determine whether the positive signal observed in the nuclei was linked to the internalization process, exogastrulation experiments were performed. Figure 6 shows an exogastrula after indirect immunofluorescence. HSP70 was not detected in the nuclei of external cells, whereas it was present in the nuclei of internal cells. From these data, it appears that the preferential nuclear location of HSP70 protein may be associated with invagination and internalization of the cells.

During neurulation, HSP70 protein remained located in the perinuclear or nuclear area of endodermic and mesodermic cells. No immunoreactivity was detected in the nuclear area of ectodermic cells. However, apparent changes in the HSP70 location became obvious in neural plate cells as neural tube formation occurred (Fig. 7A,B). Indeed, at the early neurula stage, HSP70 was not detected in nuclei of the neural plate cells, but as neurulation continued and the neurula fold formation occurred, thereby contributing to the deepening of the neural groove and to the neural tube closure, strong immunoreactivity was detected at the nuclear level of the now internalized neural tube cells (Fig. 7C,D). HSP70 was still observed in all the cell membranes.

The same immunoreactivity was detected in tailbud stages (Fig. 8).

In order to demonstrate that the immunofluorescence observed was not due to non-specific binding of the antibody, two kinds of controls were performed. We treated embryo sections with monoclonal antibody H_3F_{18} preabsorbed with the peptide antigen. In this case, no fluorescence was detected when the sections were revealed by labeled secondary goat anti-mouse IgG. Moreover, we did not observe any effect when the secondary antibody was used alone as a second control (not shown).

Discussion

Constitutive expression of inducible hsp70 mRNA during Pleurodeles embryogenesis: stability of maternal transcripts

We previously reported the existence of active transcription of a somatic heat-inducible hsp70 gene throughout *Pleurodeles* oogenesis. Furthermore, such transcription was visualized at the level of specific lateral loops of lampbrush chromosomes (Billoud *et al.*, 1993). Results from our Northern experiments and RNase protection assays enabled us to provide evidence that inducible hsp70 mRNA is also constitutively present throughout embryogenesis. These results are in disagreement with previous reports of Bienz (1984), who did not detect any hsp70 mRNA under normal conditions at any embryogenesis stage. The only cells she found to contain hsp70 mRNA without heat shock were the oocytes. In contrast, our results corroborate those of Horrell *et al.* (1987), who found that fertilized eggs and two-cell, mid-blastula and mid-gastrula embryos contained as much hsp70 mRNA as stage VI oocytes in the amphibian *Xenopus laevis*. Finally, it is worth mentioning that such accumulation of a specific subset of heat shock mRNA in normal development without heat shock has also been reported in *Drosophila* (Zimmerman *et al.*, 1983). Our results in *Pleurodeles* therefore suggest that hsp70 mRNA synthesized and stored throughout oogenesis did not turn over at fertilization, as previous-

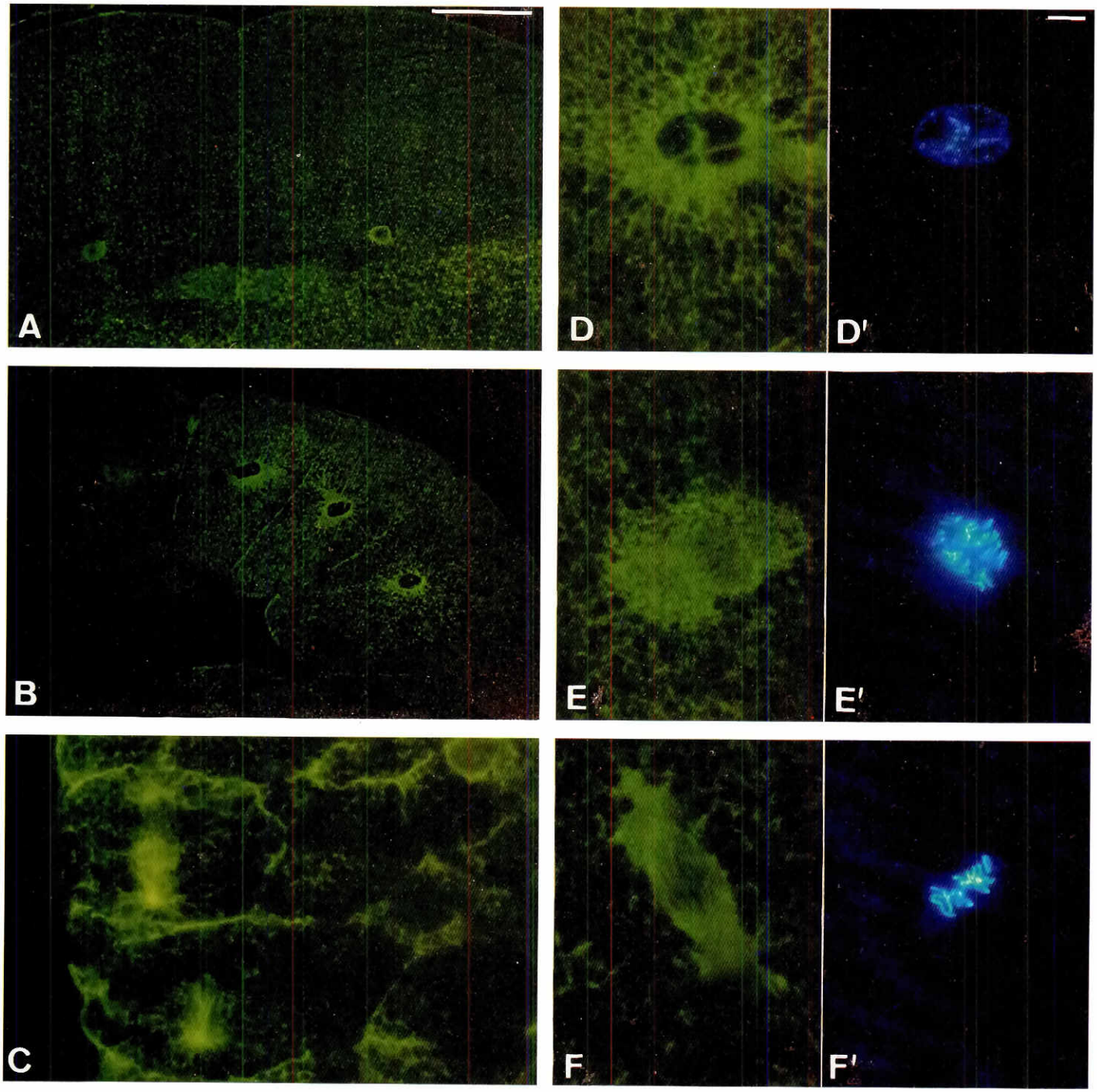

Fig. 4. Immunolocalization of HSP70-related proteins on *Pleurodeles* embryo sections showing cleavage stages. (A) *2-cell embryos showing the cytoplasmic and perinuclear location of HSP70.* **(B)** *The same location is observed in animal blastomeres.* **(C)** *Detail of a blastomere likely in mitosis.* **(D,E,F)** *Details of immunofluorescence and corresponding DAPI nuclear counterstaining of blastomere nuclei in interphase* **(D,D')** *prophase* **(E,E')** *and metaphase* **(F,F')**. *HSP70 protein is only associated with interspersed chromatin* **(D,D')** *and never with condensed chromatin of well individualized chromosomes* **(E,E';F,F')**. *Bars, 100 μm in A and B, 10 μm in C, and 5 μm in D,E and F.*

ly suggested in *Xenopus* by Bienz (1984). On the contrary, these results support the conclusion of Horrell *et al.* (1987), according to which, the maternal hsp70 transcripts persist throughout early embryogenesis under normal conditions. No profound changes in stability would therefore occur during maturation or fertilization.

HSP70-related proteins involved in embryogenesis events?

On the basis of immunocytochemical analysis, we showed that HSP70-related proteins were localized in the cytoplasm of all blastomeres at every stage of embryogenesis. Furthermore,

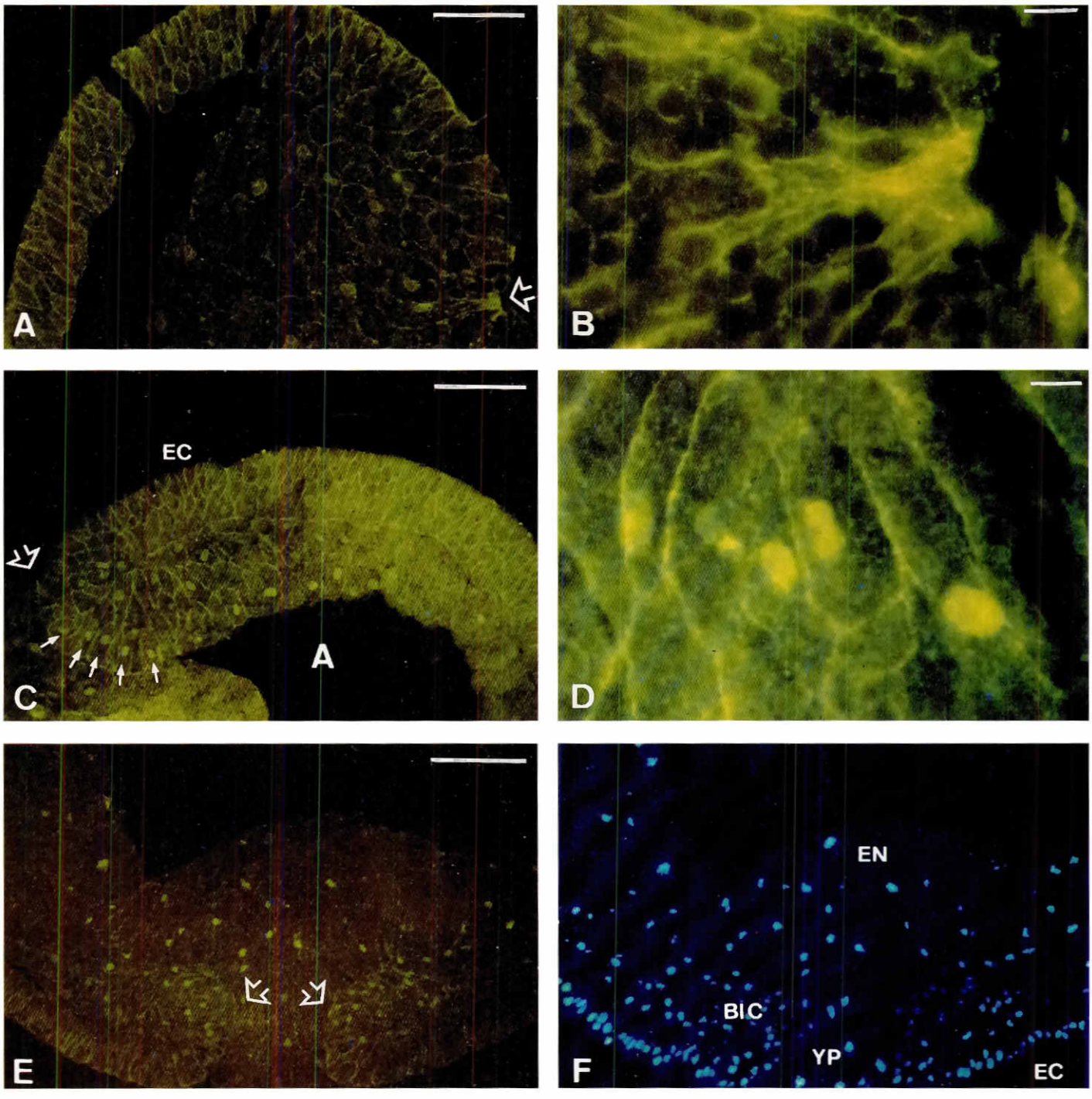

Fig. 5. Immunolocalization of HSP70-related protein at gastrula stages. (A,B) *Early gastrula stage. Immunofluorescence mainly concerns cytoplasm of all blastomeres and perinuclear regions of some. Detail of blastopore (arrow) in* **B** *shows strong fluorescence of bottle cells.* **(C,D)** *Mid-gastrula.* **(C)** *Note the nuclear labeling of cells from the involuted internal marginal zone (small arrows). In contrast, cells of the external layer do not show any nuclear labeling.* **(A)** *Archenteron. Large arrow indicates the blastopore groove.* **(D)** *Detail of involuted cells.* **(E,F)** *Small yolk plug stage.* **(E)** *Immunofluorescence showing the nuclear labeling of cells from endodermal yolk mass, and involuting cells from blastoporal collar (large arrows). Nuclei of cells from external layer visible by DAPI nuclear counterstaining* **(F)** *are not labeled. A, archenteron; YP, yolk plug; E, endoderm; Ec, ectodermal cells; Blc, blastoporal collar. Bars, 200 μm in A,C,E and F, and 20 μm in B and D.*

we showed that these proteins, up to the blastula stage, conserved the perinuclear location which had been progressively acquired during oogenesis (Billoud *et al.*, 1993). However, we pointed out that, from the gastrula, the nuclear transfer of HSP70 only involved certain cells at very precise times during normal *Pleurodeles* development.

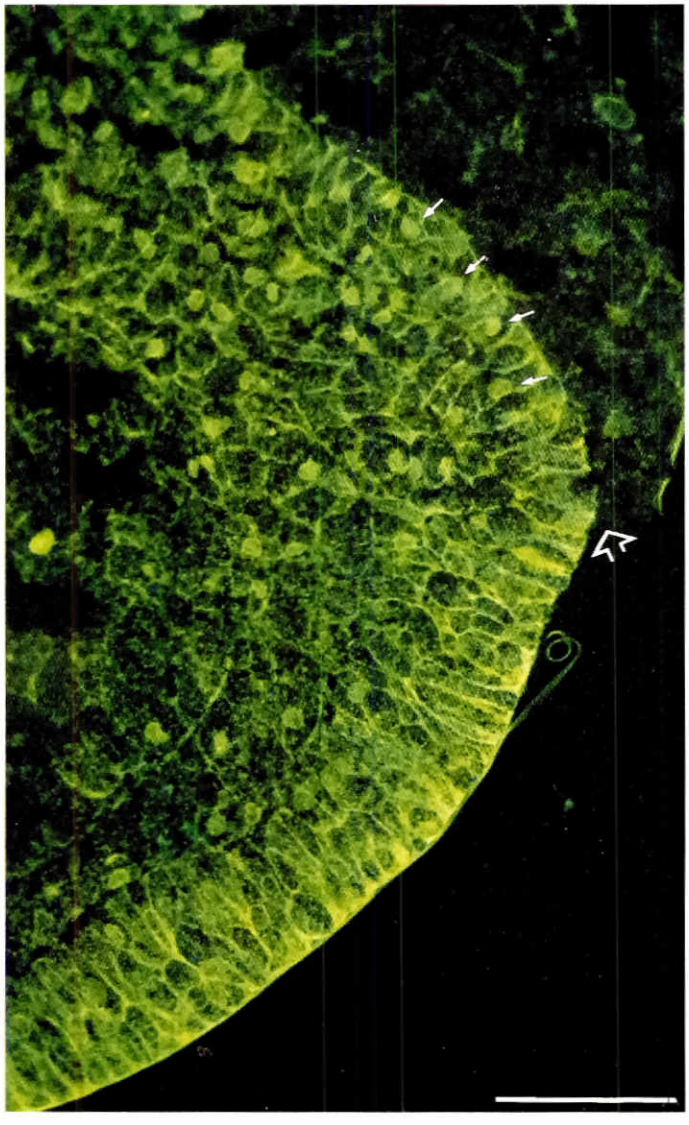

Fig. 6. Immunofluorescence of an exogastrula section: *nuclei of involuted cells are labeled (small arrows), whereas those of cells which could not invaginate are not. Large arrow : blastoporal groove. Bar, 100 µm.*

Proteins that migrate between the cytoplasm and the nucleus are of interest, since they may be involved in control processes implicated in cell maintenance, growth, replication and differentiation. In this regard, other proteins exhibit similar behavior. In *Xenopus*, the fibroblast growth factor is transferred from the cytoplasm to the nucleus in mesoderm-forming regions (Shiurba *et al.*, 1991), c-myc protein becomes nuclear after fertilization up to the gastrula stage (Gusse *et al.*, 1989), and transcription factor Xnf7 is not detectable in nuclei until the mid-blastula-gastrula stage (Miller *et al.*, 1991).

HSP70 involved in cell cycle events

With regard to the locations of HSP70 in nuclear area of blastomeres during cleavage stages, it is interesting to point out that such localization depends on the condensation state of chromatin in relation to mitotic phases. We have shown that HSP70 is associated with interspersed chromatin of the interphase nuclei, and not with well individualized chromosomes of the mitotic phases. Considering these differences in labeling, we speculate that HSP70 protein may be involved in cell cycle events. Furthermore, due to its chaperone function, HSP70 might associate with other molecules whose presence in the nuclei would be required at various periods in the cell cycle. In this respect, HSP70 protein exhibits the same behavior as other *Xenopus* and *Pleurodeles* oocyte nuclear proteins, such as XnF7, which are shifted early into the nuclei at cleavage stages (Miller *et al.*, 1991). It has also been assumed that this class of nuclear proteins may be required for cell cycle progression (Dreyer, 1987). Finally, a similar explanation has been proposed for the nuclear localization of HSP70 proteins in mammalian cells at physiological temperatures (Milarski and Morimoto, 1986; Milarski *et al.*, 1989).

HSP70 involved in cellular internalization

We reported that, at gastrulation and neurulation, HSP70 is not observed in nuclear regions of ectodermic cells. In contrast, we showed that this protein is transferred to the nuclei of specific cells at the time of their invagination and subsequent internalization. This is the case for mesodermal cells in the blastopore region of gastrula, and neurodermic cells of the neurula when the neural tube is formed. The significance of such specific nuclear transfer is unknown, but information resulting from exogastrulation experiments suggested a possible relationship between HSP70 nuclear transfer and cellular internalization phenomena. Since nuclear transfer of HSP70 no longer occurred in cells which remained external due to the exogastrulation phenomenon, it may therefore be hypothesized that HSP70 is involved in the control of nuclear activity at particular moments in embryonic development. This interpretation raises the question as to whether the role of HSP70 in control of nuclear activity is direct, via interaction with the genome, or indirect, via association with other proteins which may be required at these times and would be transported to the nuclei to control its activity. The latter possibility would be in agreement with the well known functional properties of HSP70 as a molecular chaperone which may interact with other proteins to facilitate their nuclear transport (for review, see Ellis and Van der Vries, 1991). Furthermore, results of our recent study concerning HSP70 involvement in the indirect control of transcriptional processes in *Pleurodeles* oocytes also supported this hypothesis. Indeed, these results led us to suggest that HSP70 mediates the nuclear transport of proteins directly involved in the control of transcriptional processes (Moreau *et al.*, 1994). Some of these proteins might be positive or negative transcriptional factors which would be required to regulate oocyte transcriptional activity. In a similar way, HSP70 may be indirectly involved in the control of cellular internalization processes. HSP70 might mediate the nuclear transport of proteins directly involved in the control of nuclear activity in relation to cellular internalization.

In conclusion, the behavior of HSP70 during embryonic development, and particularly its nuclear transfer to some defined cells, suggests that it is involved in the control of nuclear activity associated with important developmental events such as cleavage events and cellular internalization processes. Such a

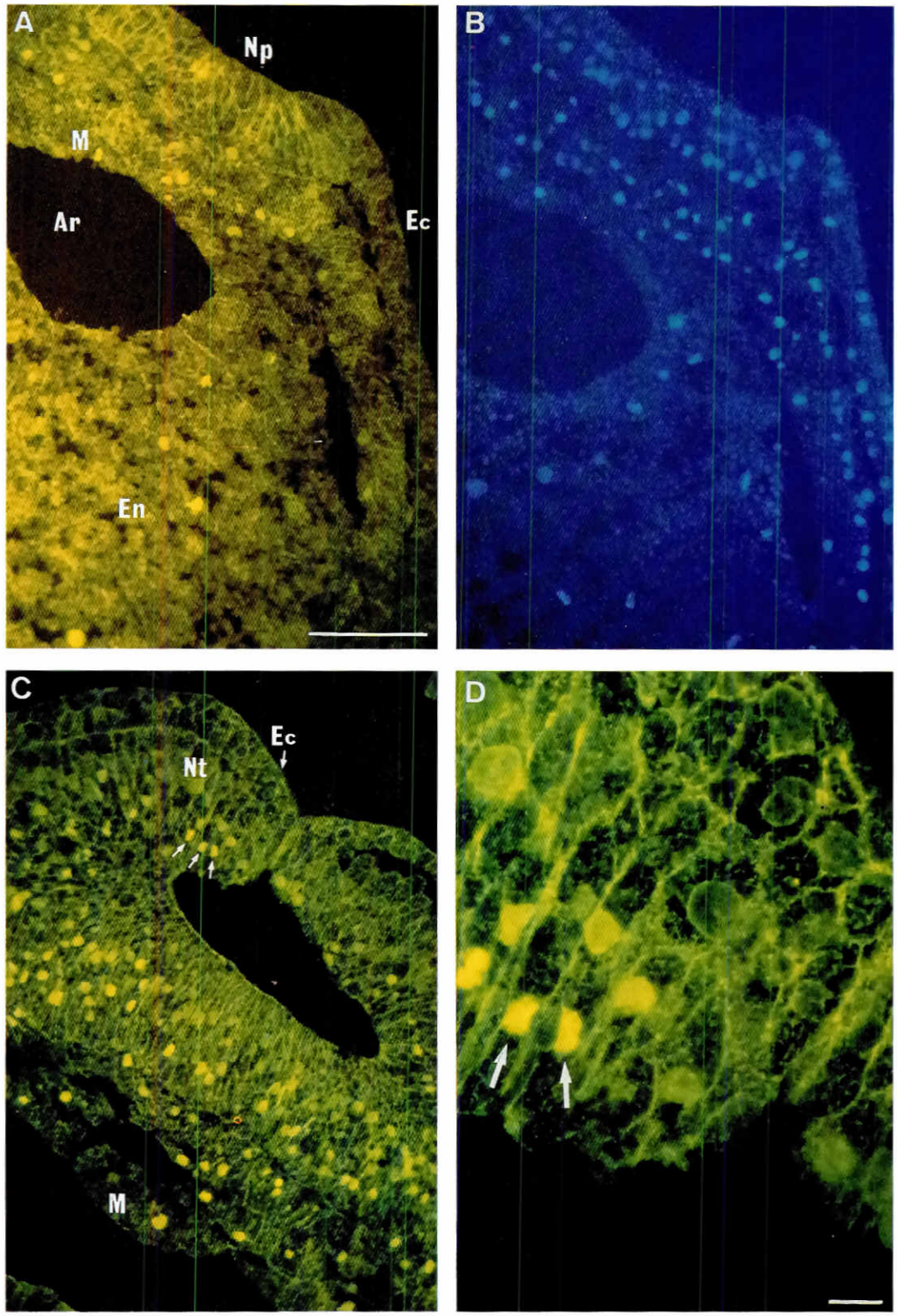

Fig. 7. Immunolocalization of HSP70-related proteins in neurula embryo sections. (A,B) *Early neurula in immunofluorescence* **(A)** *and DAPI nuclear counterstaining* **(B)**; *some nuclei of internalized endodermal and mesodermal cells are labeled, whereas those of ectodermal cells and of cells of the neural plate are not.* **(C,D)** *Late neurula in immunofluorescence. Nuclei of mesodermal cells remain labeled, whereas those of ectodermal cells remain unlabeled. In contrast, as the neural tube is forming, nuclei of the corresponding cells exhibit strong fluorescence, as indicated by small arrows in* **(C)** *and as shown in detail in* **(D)**. *Np, neural plate; M, mesoderm; En, endoderm; A, archenteron; Nt, neural tube. Bars, 150 μm in A,B and C, and 10 μm in D.*

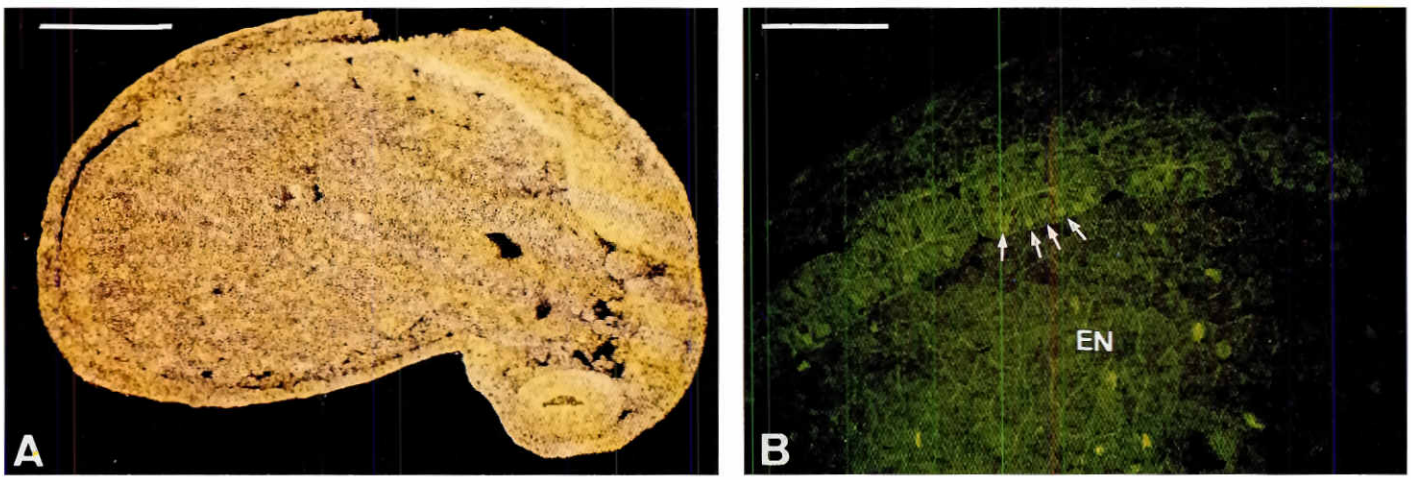

Fig. 8. Immunolocalization of HSP70-related protein in tailbud embryo section. (A) *General view of sagittal tailbud section in dark field.* **(B)** *Immunofluorescence showing the nuclear labeling of endodermal cells and mesodermal cells of somites (small arrows). Bars, 250 µm in A and 100 µm in B.*

role may be the direct consequence of its functional properties as a molecular chaperone.

Materials and Methods

Eggs and embryos

Pleurodeles waltl animals were reared in the laboratory at the physiological temperature of 20°C. Eggs and embryos were recovered by natural fertilization and spawning. Embryo stages were determined according to Gallien and Durocher (1957) updated by Shi and Boucaut (1995).

RNA, Northern hybridization and RNase protection assays

Total RNA was isolated from oocytes, eggs and embryos by the LiCl-urea method (Auffray and Rougeon, 1980). In order to estimate extraction efficiency, we added to the grinding buffer a radioactively labeled RNA (an hsp70-unrelated [^{35}S] RNA) enabling deposit on the membrane of total RNA in an amount corresponding to a fixed number of oocytes whatever the extraction yield.

Northern blotting was performed according to Sambrook *et al.* (1988). RNA samples corresponding to a fixed number of embryos determined as reported above were electrophoresed in denaturing 0.8% agarose gel, transferred onto a nitrocellulose membrane (Schleicher & Schuell BA85), and baked for 2 h under a vacuum at 80°C. The filter was prehybridized in 0.2 ml/cm^2 of 50% formamide, 6 SSC, 2xDenhardt's 1 µl/ml sonicated salmon sperm DNA for 4 h at 42°C. Hybridization was performed in the same buffer plus a denatured double-stranded [^{32}P] DNA Pw70/534 probe, the specific activity of which was typically 0.5 to 2x109 cpm/µg (Amersham's random priming kit). Washing was for 2x30 min in 1xSSC , 0.1% SDS, and for 2x30 min in 0.1% SSC, 0.1% SDS. 28S and 18S ribosomal RNAs were used as size standards to determine the approximate molecular weight of transcripts.

RNase protection analysis was performed as described by Krieg and Melton (1987) with minor modifications. cDNA fragments were cloned into pGEM7Zf(+) to direct the *in vitro* synthesis of antisense transcripts using T7 RNA polymerase (Boehringer-Mannheim, Germany) in the presence of [α-^{32}P] CTP (800 Ci/mmole, Amersham, UK). The full-length probe was purified from a 0.3 M sodium acetate, 2 mM EDTA, 0.5% SDS, 20 µg/ml tRNA polyacrylamide gel by elution at 37°C for 1 h. Following phenol chloroform extraction and ethanol precipitation, the protected fragments were resolved by electrophoresis on a 5% polyacrylamide gel and exposed to X-ray Kodak film.

Antibody

We used a mouse monoclonal antibody, m Ab H_3 F_{18}, which was raised against *Plasmodium falciparum* 72x10^3 Mr HSP-70-like protein (Mattei *et al.*, 1989). The H_3F_{18} epitope is a 16 aa sequence lying about 160 residues upstream of the protein C terminus.

Protein extraction and immunoblotting

Batches of 10 embryos at each stage were homogenized in Tris-EDTA buffer (Chen and Stumm-Zollinger, 1986). The homogenate was centrifuged for 10 min at 10,000g and proteins of the supernatant were precipitated overnight at -20°C by 9 vol of ethanol. Total oocyte proteins were separated in 10% polyacrylamide gels, either in one (Laemmli, 1970) or two dimensions (O'Farrell, 1975), and then electrophoretically transferred to an Immobilon-PVDF membrane (Millipore). Blots were treated with antibody H_3F_{18} at room temperature. After washing, they were incubated with ^{125}I-labeled (specific activity 3000 Ci/mmole, Amersham) antimouse antibody.

Immunolocalization

Immunocytochemistry experiments were carried out by indirect immunofluorescence: 7.5 µm thick sections of embryos at every stage were prepared as previously described (Moreau *et al.*, 1986), fixed with Romeis fixative and embedded in polyester wax (Hausen *et al.*, 1985). Sections were overlaid with 1% BSA in PBS for 30 min and then incubated with the monoclonal antibody H_3F_{18} against HSP70 (1:200 dilution in 1% BSA, PBS). After three washes in PBS, slides were incubated with a fluorescein isothiocyanate-conjugated goat anti-mouse BSA (Miles Scientific, Paris), washed and mounted in Mowiol.

Acknowledgments

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