Int. J. Dev. Biol. 42: 633-636 (1998)

Short Contribution

Cell-cycle-dependent nuclear translocation of HSP70 in amphibian embryonic cells

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ABSTRACT Using immunocytochemical methods, we analyzed the localization of the HSP70 protein constitutively expressed during embryogenesis in the amphibian *Pleurodeles waltl*. Our results provide evidence for nuclear transfer of the protein during gastrulation, and particularly for predominant nuclear labeling in gastrula internalized cells. Using two inhibitors of DNA replication –hydroxyurea (HUA) and aphidicolin– or/and an inhibitor of transcription –actinomycin D– applied to embryos, we demonstrated that nuclear transfer of HSP70 is related to the transcriptional activity of the cells during the early S phase of the cell cycle.

KEY WORDS: *cell cycle, transcription, nuclear translocation, HSP70, amphibian embryos*

The role of stress-70 proteins during the heat-shock response has been extensively studied for many years (for review, see Lindquist and Craig, 1988; Craig and Gross, 1991), but their importance in normal cellular processes was described only more recently. Indeed, HSP70 is expressed constitutively and abundantly in the absence of any stress, and is essential for cell viability under normal growth conditions. It behaves as a molecular chaperone, and is involved in promoting protein folding, transport, and assembly in both eucaryotic and procaryotic systems (for review, see Ellis and Van der Vies, 1991; Gething and Sambrook, 1992). HSP70 cognate protein (HSC70) is required for translocation of nascent proteins into the endoplasmic reticulum and mitochondria (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Kang *et al.*, 1990; Scherer *et al.*, 1990; Vogel *et al.*, 1990). HSC70 also interacts with NLS-containing proteins in the cytoplasm before their nuclear import (Imamoto *et al.*, 1992). HSC70 is known to be localized in both cytoplasm and nucleus, and to shuttle between the nucleus and the cytoplasm (Mandell and Feldherr, 1990). In Hela cells, the protein becomes concentrated in the nucleus of early S phase cells, but it is diffusely distributed throughout the cytoplasm and the nucleus during the remainder of the cell cycle. In hepatocytes from a regenerating rat liver, Ohmori *et al.* (1990) described a simultaneous increase in hsp70 and nucleoline during the G_1 phase. In addition to this HSP70 cell cycle regulation, HSP70 interacts with other components in the cell, and these interactions are cell-cycle-specific (Milarski *et al.*, 1989).

In the amphibians *Xenopus lævis* and *Pleurodeles waltl*, we previously described nuclear transfer of HSP70 to some embryonic cells, and in particular to cells internalized during gastrulation (Herberts *et al.*, 1993; Angelier *et al.*, 1996). However, the significance of such transfer remains unexplained. Is this nuclear transfer related to the process of internalization, linked to a defined phase of the cell cycle, or related with any other specific functions?

In order to address this question, we first used two inhibitors of DNA replication: hydroxyurea (HUA) (Gilman *et al.*, 1980) and aphidicolin (Ikegami *et al.*, 1978). When applied to stage 10 (early gastrula) *Xenopus* embryos, HUA solution is known to allow gastrulation to occur (Harris and Hartenstein, 1991). We applied HUA solution to early gastrula of *Pleurodeles* embryos. The gastrulation approximately proceeded and subcellular localization of HSP70 in the treated embryos enabled us to relate nuclear transfer to the replication phase. Second, we used actinomycin D and finally, both the replication and transcription inhibitors were successively used. Thus we were able to link the nuclear location of HSP70 to a role played by this protein in the transcription processes which occurred during the replication phase.

Distribution of HSP70 in embryos was investigated by immunofluorescence on sections using antibody H_3F_{18} (see Experimental Procedures). In order to demonstrate that the immunofluorescence we observed was not due to non-specific binding of the antibody, two kinds of control were performed. First, we treated embryo sections with another mouse monoclonal antibody: anti-mouse glial fibrillary acidic protein (GFAP, Bœhringer Manheim). After treatment with labeled secondary goat anti-mouse IgG, no fluorescence was ever detected. In addition, we did not observe any effect when secondary antibody was used alone.

Distribution of HSP70 protein during the gastrula stage

We previously described the localization of HSP70 during *Pleurodeles* embryogenesis (Angelier *et al.*, 1996), and espe-

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0214-6282/98/$10.00

Printed in Spain

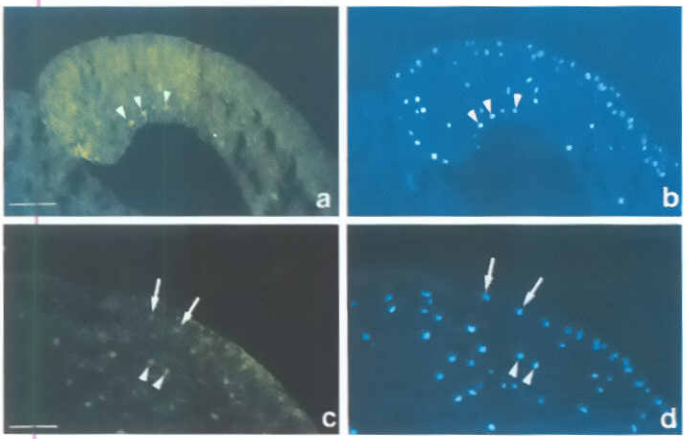

Fig. 1. Immunolocalization of HSP70 protein on Pleurodeles embryo sections showing a gastrula stage and Hœscht counterstaining. (a,b) *Detail of the region of the dorsal blastoporal lip of an early gastrula. The nuclei of the internalized cells are predominantly labeled (arrowheads).* **(c,d)** *Detail of the region of the dorsal blastoporal lip of an early gastrula. The nuclei of the internalized cells (arrowheads) as well as those of the external cells (arrows) are labeled. Bar , 200 μm.*

cially during the gastrulation stage. On the basis of immunocytochemical analysis, we showed that HSP70 was localized in the cytoplasm of cell blastomeres. HSP70 was not observed in nuclear regions of ectoderm cells. In contrast, we showed that the protein was transferred to the nuclei of specific cells at the time of their invagination and subsequent internalization (Fig. 1a,b). However, today, we are able to assert that in 20% of the embryo sections we observed, all cells –both internalized and external ones– were concerned by the nuclear transfer of HSP70 (Fig. 1b,c). Thus, in the nuclei of gastrula cells, HSP70 is always present in the internalized cell nuclei. However, in the external cells nuclei, the protein was weakly present except in 20% of sections.

These observations led us to the hypothesis that nuclear transfer of HSP70 was related to special phase of the cell cycle. In order to check such hypothesis, two DNA synthesis inhibitors were first used to block cell division (see Experimental Procedures). In a second step, we used actinomycin D in order to inhibit transcription processes. In a third step, the three inhibitors were used simultaneously.

Distribution of HSP70 protein in embryos treated with HUA or/ and actinomycin D

In embryos resulting from stage 8a gastrula treated with DNA synthesis inhibitors (hydroxyurea and aphidicolin), we observed that gastrulation went on. Gastrulation occurred normally up to the midgastrula stage, and often even up to the vitellus plug. At that moment, gastrulation stopped and neurulation never occurred. Sections of such embryos treated with anti-HSP70 provided evidence for a significant labeling in all the nuclei of peripheral cells, as well as in those which were internalized (Fig. 2a,g). These cells exhibited the same dispersed state of chromatin as shown by Hœscht counterstaining (Fig. 2b,h).

In embryos resulting from stage 8a gastrula treated with actinomycin D, we observed a nuclear staining in the internalized cells, and a very weak staining in the nucleus of external cells (Fig. 2c,d). This was observed in every embryo treated with actinomycin D.

In embryos resulting from stage 8a gastrula treated with HUA associated with actinomycin D, the nuclear staining was observed only in the internalized cells (Fig. 2e,f). As compared to the HUA-treated embryos, staining of the external cell nuclei completely disappeared.

We previously suggested that the behavior of HSP70 during embryonic development, and particularly its nuclear transfer to some defined cells, was related to an involvement in the control of nuclear activity associated with important developmental events such as cleavage events and cellular internalization processes. In the present report, our results led us to suggest that such a nuclear localization in embryonic cells may be related at least to transcriptional processes occurring during the replication phase.

During early embryogenesis, from the two-cell to the midblastula stages, although HSP70 was localized on chromatin (Angelier *et al.*, 1996), we never detected significant nuclear transfer of the protein. In contrast, during gastrulation, HSP70 nuclear transfer occurred. This transfer primarily concerned internalized cells and, in some cases, all the gastrula cells. Such a nuclear transfer was also observed in late blastula cells, both in the animal and vegetal hemispheres (data not shown).

It is known that, in the amphibian embryo, the early cleavage cycle results from rapidly alternating M and S phases, with no appreciable G_1 or G_2 phases (Graham and Morgan, 1966). During this period, the zygotic genome cannot be transcribed, since chromosome condensation in the M phase is incompatible with transcriptional processes and rapid replication in the S phase is also incompatible with the establishment of stable transcription complexes (Kimelman *et al.*, 1987). Transcriptional dormancy during this period involves an overabundance of a putative factor that prevents a G phase in the cell cycle by rapidly triggering the onset of mitosis after the S phase. After 12 cell divisions, when a critical nucleus-to-cytoplasm ratio is reached, titration of this factor results in a slower cell cycle which allows transcription to occur (Newport and Kirschner, 1982a,b; Kimelman *et al.*, 1987).

Newport and Kirschner (1982a) reported several changes in the cell cycle after MBT: i) the onset of G_1 and G_2 phases; ii) lengthening of the S phase; and iii) transcription of the zygotic genome. Furthermore, some cells acquire motility. Therefore, we hypothesize that the nuclear transfer of HSP70 that we observed after mid-blastula and during gastrulation may be correlated with at least one of these events.

In order to address this question, we dissociated these events. We blocked the cell cycle of gastrula cells in the S phase using two inhibitors of DNA synthesis, hydroxyurea and aphidicolin. We know that hydroxyurea applied at the MBT phase blocks the cell cycle in the S phase by inhibiting replication (Newport and Dasso, 1989). Maurer-Schultze *et al.* (1988) demonstrated that L1210 tumor cells treated with hydroxyurea enter the S phase at about a normal rate and accumulate in the early S phase due to a dose-dependent inhibiting effect of HUA on DNA synthesis. We combined hydroxyurea with aphidicolin because it worked quickly (within 2 h), while aphidicolin worked more slowly (within 4-6 h). However, aphidicolin blocked DNA synthesis more completely and irreversibly than did hydroxyurea (Harris and Hartenstein, 1991). We therefore assume that the embryonic cells we observed were blocked in the early S phase of the cell cycle. Our results show that the early gastrula treated with these two inhibitors proceeded normally up to the late gastrula stage without cell division.

In amphibian embryos, the major differences between the S phase prior to and after MBT is the length of the phase and the beginning of zygotic genome transcription. This transcriptional activity may occur during the G1, S or G_2 phase of the cell cycle.

In our experimental study, all embryonic cells were blocked in the early S phase of the cell cycle; in this case, HSP70 was transferred to cell nuclei of the embryos. The abundance of HSP70 in the nuclei may be therefore related to either replication or transcriptional processes.

Expression of some HSP is under developmental control in the sea urchin (Roccheri *et al.*, 1981), *Drosophila* (Thomas and Lengyel, 1986), *Xenopus* (Heikkila *et al.*, 1991) and the mouse (Bensaüde and Morange, 1983). These HSP probably play a role in growth and differentiation processes. Walsh *et al.* (1993) demonstrated that the heat shock gene may function as a cell cycle regulator in neuroectoderm induction and differentiation of rat embryos. They also showed that thermotolerance is associated with cell cycle delay in the S phase and specific hsp expression at the G_1/S and S/G_2 boundaries.

In human cells, the HSP70 protein is expressed at physiological temperatures in a cell cycle-dependent manner (Milarski and Morimoto, 1986). In Hela cells, expression of HSP70 is restricted to the G_1/S boundary. Indirect immunofluorescence with HSP70-specific monoclonal antibody reveals that the protein becomes concentrated in the nucleus of early S phase cells (Milarski and Morimoto, 1986).

Our results are in good agreement with these observations. Indeed, we observed nuclear transfer of HSP70 in the early S phase. In addition, we can assume that this transfer is linked to a transcriptional activity of the cell, since in the S phase without transcription, when embryos were treated with actinomycin D, this transfer did not occur, at least for the external cells.

During gastrulation, the nucleus of internalized cells was preferentially labeled by HSP70 protein antibodies whatever the inhibitor treatment to which they were submitted. We believe that for these cells, HSP70 is undoubtedly in relation with transcriptional activity during the S phase. However, HSP70 in these cells is likely to interact with other functions which are still not elucidated.

Our present results are in good agreement with our previous studies which demonstrated that HSP70 may interfere with transcriptional activity in the amphibian oocyte (Moreau *et al.*, 1994). However, Imamoto *et al.* (1992) reported that RNA synthesis went on normally when an HSP70 antibody was injected into mammalian cells.

HSP70 proteins are known to be chaperone molecules able to associate with a number of newly synthesized proteins (Beckmann *et al.*, 1990). They are also known to stabilize the unfolded state of proteins (Beckmann *et al., 1990;* Kang *et al.*, 1990; Mizzen *et al.*, 1991; Gething and Sambrook, 1992). They are involved in transport of proteins to the nucleus (Imamoto *et al.*, 1992; Shi and Thomas, 1992; Okuno *et al.*, 1993). The results of our present study led us to suggest that HSP70 may play a role in control of RNA transcriptional activity during the S phase in embryonic cells. Further studies on proteins whose nuclear transport depends on HSP70 are required to determine the nature of this control.

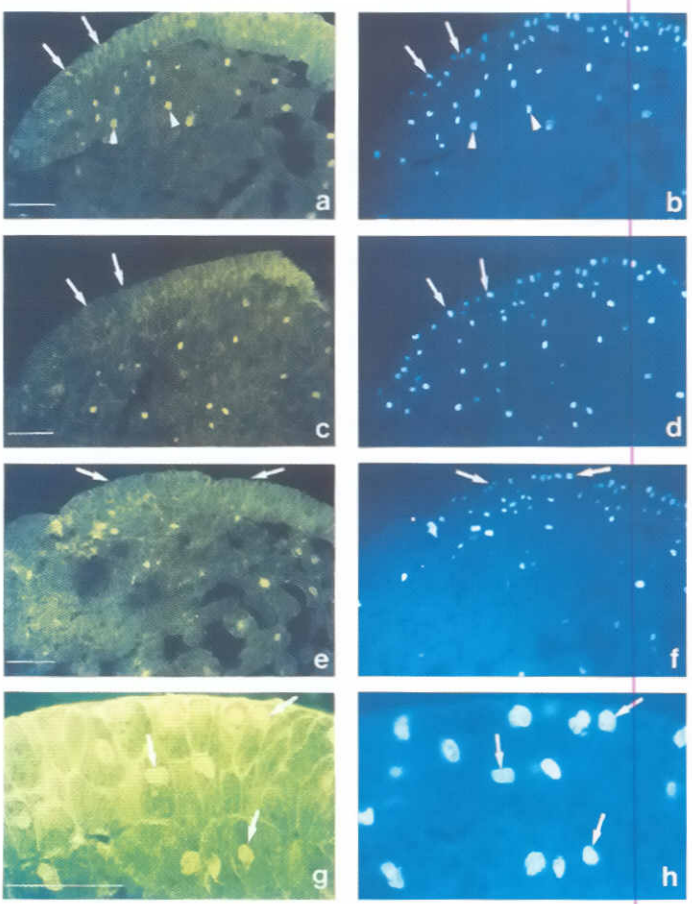

Fig. 2. Immunolocalization of HSP70 protein on Pleurodeles embryo sections submitted to inhibitors of replication (hydroxyurea and aphidicolin) or/and inhibitor of transcription (actinomycin D) from early gastrula stage. *Immunofluorescence and Hœscht counterstaining observed in the region of the dorsal blastorporal lip.* **(a,b)** *After HUA treatment, all the nuclei are fluorescent in external (arrows) and internalized cells (arrowheads).* **(c,d)** *After actinomycin D treatment, the nuclei of internalized cells are fluorescent. Those of external cells show no preferential staining (arrows).* **(e,f)** *After HUA plus actinomycin D treatment, the fluorescence is no longer observed in the nuclei of external cells (arrows) as compared with the situation in (a, b,).* **(g h)** *High magnification after HUA treatment. The translocation of HSP70 concerns all the nuclei (arrows). Bar, 200 μm.*

Experimental Procedures

Embryos

Pleurodeles waltl embryos were obtained by *in vivo* fertilization. Their jelly was manually taken off. Unless otherwise indicated, embryos were maintained in 10% modified Barth's solution (MBS) (Gurdon, 1976). The embryo stages were determined according to Gallien and Durocher (Gallien and Durocher, 1957), and Shi and Boucaut (1995).

HUA treatment

When the embryos reached the early gastrula stage (8a), they were injected with 50 nl of a solution of 40 mM hydroxyurea (a blocker of ribonucleotide diphosphate reductase, an enzyme that catalyzes the reductive conversion of ribonucleotide to desoxyribonucleotide and 3 mM aphidicolin (an inhibitor of eukaryotic DNA polymerase α (Harris and Hartenstein, 1991). The gastrula were pipetted into a solution of 20 mM hydroxyurea and 150 μM aphidicolin in 10% MBS solution (adjusted to pH 7.4). HUA-treated embryos were kept continuously in HUA solution. Embryos from the same batch maintained in 10% MBS were used as controls.

Actinomycin D treatment

In order to inhibit transcription, stage 8a embryos were microinjected with 50 nl of a 50 μg/ml actinomycin D solution. The embryos were then maintained in 10% MBS solution.

Antibody

We used mouse monoclonal antibody $mAbH_3F_{18}$ raised against a *Plasmodium falciparum* 72x103 Mr HSP70-like protein (Mattei *et al.*, 1989). The specificity of this antibody was checked by binding it to two-dimen-

sional electrophoretically separated and blotted *P. waltl* oocyte polypeptides (Billoud *et al.*, 1993).

Immunolocalization

For immunolocalization, 7.5 μm thick sections of embryos fixed with Romeis fixative (25 ml saturated $HgCl_2$, 20 ml TCA, 15 ml 37% formaldehyde) and embedded in polyester wax (Hausen *et al.*, 1985) were prepared as already described (Moreau *et al.*, 1986). They were incubated for 1 h with mAbH3F1$_8$ antibody (1/100 dilution) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). After 3 washes in PBS, slides were incubated with a fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Miles Scientific, Paris) supplemented with a Hœchst counterstain (Sigma-Aldrich) (4 μg/ml), washed and mounted in Mowiol.

Acknowledgments

This work was supported by fellowships from the French Research Ministry (MESR) and by grants from the CNRS, the University Pierre et Marie Curie (Paris) ACC n° 4 and the Cancer Research Association (ARC n°6309). We thank Dr. D. Mattei and Dr. P. Dubois for the gift of mAb H_3F_{18} (Pasteur Institute, Paris).

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Received: August 1997
Accepted for publication: January 1998