Experimental Cell Research **260,** 222–232 (2000)
doi:10.1006/excr.2000.5003, available online at http://www.idealibrary.com on IDEAL®

HSP70 Is Involved in the Control of Chromosomal Transcription in the Amphibian Oocyte

Charlotte Delelis-F. Corporeau, Nicole Angelier, and May Penrad-Mobayed[1]

Groupe Gènes et Développement, UMR 7622, Université P. et M. Curie, CNRS, Laboratoire de Biologie Moléculaire et Cellulaire du Développement, Bât. C, 6ème étage, Case 16, 9 quai Saint-Bernard, 75252 Paris cedex 05, France

The amphibian oocyte represents an excellent model for the study of transcription regulation. Indeed, any modification of transcriptional activity is directly reflected in lampbrush chromosome structure by concomitant morphological changes. Previous studies have led to the hypothesis of a putative role for heat-shock proteins HSP70 and/or HSC70 in transcriptional processes in the oocyte. In order to dissect out the relative role of HSP70 or HSC70 in these processes, we used an oligo-antisense strategy to specifically inhibit the function of the targeted protein. Effects of hsc70 and hsp70 antisense oligodeoxynucleotides were analyzed in terms of both mRNA quantity and protein synthesis. Their effects on oocyte transcription were analyzed at the level of structural organization of lampbrush chromosomes and nucleolar transcriptional activity. Our results show that specific inactivation of hsc70 mRNA by hsc70 antisense oligos led to a reversible inhibition of lampbrush chromosome transcription. However, such reversible inhibition of transcription is considered non-sequence specific since it is also induced by any oligo. In contrast, specific inactivation of hsp70 mRNA by hsp70 antisense oligos, which is correlated with a drop of HSP70 neosynthesis, results in an irreversible inhibition of lampbrush chromosome transcription. Furthermore, our results show that the inactivation of hsp70 or hsc70 mRNAs does not affect nucleolar transcription. Such data suggest a role for HSP70 in the control of chromatin modifications related to RNA polymerase II transcriptional activity.

Key Words: **amphibian; oocyte; HSP70; HSC70; transcription.**

INTRODUCTION

Heat shock proteins (HSPs) are multifunctional and are among the most highly conserved set of proteins. *Hsp/hsc70* genes have been reported in a wide variety of eukaryotic cells from yeast, *Drosophila*, amphibians, mice, and humans [1]. In higher organisms, *hsp70* belongs to a multigenic family that includes the transiently stress-inducible form(s) of HSP70 proteins involved in regulation of the cellular response to various types of stress, and one or more cognate protein(s) HSC70 constitutively expressed under normal conditions, during cell cycle and differentiation [for a review, see 2].

HSC70(s) and HSP70(s) were identified in oocytes and embryos of two amphibians, *Xenopus laevis* and *Pleurodeles waltl* [3–9]. In *Pleurodeles waltl*, both cognate HSC70 and inducible HSP70 were shown to be constitutively expressed in oocytes as well as in embryos [3, 7], and several forms of HSP/HSC70 were shown to be stored in a large amount in the *P. waltl* oocyte [9]. Furthermore, in the *P. waltl* oocyte, an indirect role of HSP/HSC70 in the control of polymerase II transcription was suggested. Indeed, injection of anti-HSP/HSC70 monoclonal antibody into the oocyte cytoplasm led to retraction of lampbrush chromosomes, reflecting inhibition of transcription. In contrast, no modifications were observed when the antibody was directly microinjected into the nucleus [10]. These results suggested that HSP/HSC70 mediates the nuclear transport of proteins involved in the lampbrush chromosome transcriptional processes. However, no evidence was provided for a specific role of HSP70 or HSC70 since this antibody recognized an epitope common to both HSP70 and HSC70.

In order to determine whether such a role was restricted to either HSP70 or HSC70, we used an oligo-antisense strategy to specifically inhibit the function of one targeted protein. Injection of an antisense oligo induces *in vivo* inactivation of one existing mRNA and inhibits the corresponding protein function [11–14]. Such an oligo-antisense strategy has already been used in the amphibian oocyte [14–18]. We analyzed the effects of specific hsp70 and hsc70 antisense oligodeoxynucleotides in terms of both mRNA quantity and protein synthesis. We also analyzed their effects on oocyte transcription using light microscopy at the

[1] To whom correspondence and reprint requests should be addressed at Institut Jacques Monod, CNRS, Universités Paris 6 et Paris 7, 2 place Jussieu, 75251 Paris CEDEX 05, France. Fax: (+33) 1 44 27 57 16. E-mail: penrad@ijm.jussieu.fr.

0014-4827/00 $35.00

structural organization level of lampbrush chromosomes and by autoradiography. Indeed, due to their large size and particular organization, amphibian lampbrush chromosomes constitute an excellent model for the study of transcription. Lateral loops are the sites of RNA polymerase II transcriptional activity [19]; the newly synthesized RNA binds to proteins to form ribonucleoprotein (RNP) complexes [for a review, see 20]. Any modification occurring at the trancriptional level is immediately reflected in the structure of lampbrush chromosomes by concomitant morphological changes. Thus, inhibition of RNA transcription induced by drugs such as actinomycin D or α-amanitin led to rapid release of RNP transcripts, loop retraction, and chromosomal condensation [21; for a review, see 20]. When drugs were removed and transcription resumed, the normal loop structure was restored. The significance of lateral loops has recently been explained by a biophysical polymer model, in which the development of the loop axis requires forces produced by RNP transcripts [22].

Here we provide evidence for specific involvement of inducible HSP70 in the control of chromosomal decondensation related to transcriptional processes. Although HSP70 is stored throughout oogenesis, such a role only concerns the neosynthesized fraction of HSP70. In contrast, HSC70 was not found to be implicated in this process. Furthermore, neither HSP70 nor HSC70 was shown to be involved in nucleolar transcription.

MATERIALS AND METHODS

Plasmid construction. *P. waltl hsp70* cDNA was characterized by Billoud *et al.* [3]. The entire 2234-bp *hsp70* cDNA was subcloned in a modified pBluescript-RN3 expression vector [23]. This vector contains the 5′ and 3′ untranslated regions of the *X. laevis* globin gene (80 and 200 bp, respectively) responsible for synthetic mRNA *in vivo* stabilization. In order to generate an *hsp70* myc-tagged cDNA containing six copies of the myc epitope, we amplified 1954 bp of *hsp70* cDNA by polymerase chain reaction to generate the 5′-end *Stu*I and the 3′-end *Xba*I restriction enzyme sites. Then, this *hsp70* cDNA was cloned in the pCS2$^+$MT vector in phase [24, 25].

In vitro transcription and translation. Sense wild-type *hsp70* or sense *hsp70* myc-tagged mRNAs were transcribed *in vitro* with either bacteriophage T_3 or Sp_6 RNA polymerase (Riboprobe system) according to the manufacturer's instructions. Both synthetic mRNAs were transcribed and translated *in vitro* using rabbit reticulocyte lysate (Rabbit Reticulocyte Lysate Systems, Promega) with [^{35}S]methionine. Aliquots of translation products were electrophoresed in monodimensional SDS 10% polyacrylamide gel. Dried gels were autoradiographed on Amersham β-Max film. The molecular mass of the translation products was determined with reference to the molecular mass markers.

Oligos. Single-stranded 20-mer oligodeoxyribonucleotides were synthesized by GENSET. Their sequences and orientation are indicated in Table 1 (see above). Several pairs of antisense (hspAS1, hspAS2; hscAS1, hscAS2) and sense oligos (hspS1, hspS2; hscS1, hscS2) were complementary or identical to the corresponding *hsc70* mRNA or *hsp70* mRNA. A search within the nucleotide BISANCE database did not reveal any identity of oligos with genes other than corresponding *hsp70* or *hsc70* genes. (αV), an oligo unrelated to any heat-shock mRNA, was identical to αV integrin mRNA [26].

TABLE 1

Name	Sequence	Position on cDNA (nt)
hspAS1	5′ GACATCTTCTGCAGTGCTGC 3′	+5; −15
hspS1	5′ GCAGCACTGCAGAAGATGTC 3′	−15; +5
hspAS2	5′ AGTAGGTCCAGTGCTGGAGC 3′	+2194; +2175
hspS2	5′ GCTCCAGCACTGGACCTACT 3′	+2175; +2194
hscAS1	5′ CATTGTCCGTGCTGATCTCG 3′	+3; −17
hscS1	5′ CGAGATCAGCAGGCACAATG 3′	−17; +3
hscAS2	5′ TGAGCCACCGCTGCCAGCTG 3′	+1947; +1928
hscS2	5′ CAGCTGGCAGCGGTGGCTCA 3′	+1928; +1947
αV	5′ TCCCGAGTGCTGTCTGAATG 3′	−17; +3

Oligo injection experiments. *P. waltl* (Amphibian, Urodele) females were raised at 20°C in our laboratory. Ovaries were surgically removed from anesthetized females (0.1% MS 222, Sandoz) and oocytes were defolliculated as previously described [27]. After incubation overnight at 20°C in culture medium OR2 [28], healthy stage V–VI oocytes [29] were selected and injected with 12 to 50 nl of single-stranded oligodeoxynucleotides diluted in H_2O at a concentration of 1 ng/nl. After injection, the oocytes were maintained at 20°C in OR2. Five, 24, and 48 h after injection, oocytes were collected and assayed for lampbrush chromosome spreads, mRNA quantitation, or protein analysis. Ten experiments were monitored, each including at least 400 oocytes taken from the same female.

Recovery experiments. Twenty-four hours after hsp70 antisense oligo injection (hspAS1, hspAS2), oocytes were injected again with 25 nl of synthetic *hsp70* mRNA or *hsp70* myc-tagged mRNA solution [1 μg/μl]. Oocytes were then maintained for 24 h at 20°C in OR2 and chromosomal activity was checked.

Oocyte maturation assays. Oocytes injected 24 h previously with hsp70 or hsc70 sense or antisense oligos or control oocytes were incubated overnight in OR2 medium containing [10 μg/ml] progesterone (Sigma). The "white spot" appearance was checked, indicating that oocyte maturation was proceeding. Germinal vesicle breakdown was ascertained by the absence of the germinal vesicle determined by dissection of oocytes after 10 min fixation in 10% trichloroacetic acid (TCA).

Lampbrush chromosome preparations for light microscopy. Lampbrush chromosomes were prepared as described [30]. Nuclei from stage V–VI oocytes of *P. waltl* were manually isolated in sterile physiological medium containing 72 mM KCl, 25 mM NaCl buffered to pH 7.2 with 10 mM Tris-HCl, 0.01 mM $MgCl_2$, and 0.01 mM $CaCl_2$ [31]. They were then transferred to an observation chamber. The nuclear membrane was removed using forceps and needles. After the nuclear content was spread, the chamber was centrifuged at 2200*g* for 30 min at 4°C. Then, chromosome preparations were fixed in 70% ethanol for 30 min and dehydrated through an ethanol series. Lampbrush chromosomes were then observed with phase-contrast microscopy.

Autoradiography for light microscopy. Oocytes were incubated for 24 h in OR2 containing [^{3}H]uridine [500 μCi/ml] and then washed in three baths of culture medium. Chromosome preparations were done as previously described. They were then processed for autoradiography [21]. Nuclear spreads including chromosomes and nucleoli were fixed for 30 min in 2% paraformaldehyde in phosphate buffer at pH 7.2 and dehydrated through an ethanol series. They were dipped in NTB2 emulsion (Kodak) diluted 1/1 with distilled water, left to expose at 4°C for 8 days, and developed at 15°C in Kodak D19

developer. The preparations were stained with Coomassie blue (0.1% in 50% methanol, 10% acetic acid) for 10 min as described [32].

RNA extraction and RT-PCR. Total RNA was extracted from stage V–VI oocytes using the RNA Insta-pure kit method (Eurogentec). Of total RNA 250 ng was used for the reverse-transcriptase reaction with oligo(dT) primers [25 μg/μl] according to the manufacturer's instructions (Gibco BRL). Either 1234 bp of *hsp70* cDNA or 740 bp of *hsc70* cDNA in the 3′ coding region or 692 bp of *alphaV integrin* cDNA were amplified by the polymerase chain reaction using AmpliTaq-DNA polymerase (Perkin-Elmer). PCR experiments were performed under conditions in which saturation was not reached: 30 thermocycles were performed for 30 s at 93°C, 30 s at either 53, 58, or 62°C, respectively, and 90 s at 72°C. PCR products were electrophoresed in 1% TBE agarose gel, blotted onto a nylon membrane (Nytranplus, Schleicher and Schull) and hybridized with the corresponding ^{32}P-labeled probes. Hybridization was carried out at 68°C in 6× SSC, 2× Denhardt's, 0.1% SDS. Blots were washed 20 min in 1× SSC, 0.1% SDS at room temperature, and 3 × 20 min at 68°C in 0.2× SSC, 0.1% SDS, and then autoradiographed on Amersham MP film. Hybridization signals were quantitated using the Phosphoimager system (Molecular Dynamics, Sunnyvale, CA) and the supplied software (Image Quant; Molecular Dynamics).

Protein extraction and SDS polyacrylamide gel electrophoresis. Oocytes were incubated for 4 h in OR2 containing [^{35}S]methionine [500 μCi/ml] and then washed in three baths of culture medium. Nuclei from stage V–VI oocytes were manually extracted from oocytes and placed in Tris-ethanol solution (Tris [6 mM], $MgCl_2$ [3 mM], and 70% ethanol). Nuclear proteins were precipitated overnight at −20°C in 6 mM Tris, 45% ethanol, 45% glycerol, centrifuged 5 min at 12,000*g*, and then dissolved in "O" electrophoresis buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% dodecyl sodium sulfate, [62.5 mM] Tris, bromphenol blue). Two-dimensional electrophoresis [33] with equilibrium pH gradient was performed using ampholines 3-11 (Bio-Rad) in the first dimension, followed, in the second dimension, by electrophoresis in SDS-10% polyacrylamide slab gel, as described by Laemmli [34]. Gels were dried and autoradiographed on Amersham β-Max film.

Immunodetection on Western blot. In vitro translation products or oocyte proteins separated by two-dimensional electrophoresis were transferred to an Immobilon-PVDF membrane (Millipore) [35]. Then, blots were incubated for 1 h at 42°C in 1% BSA in PBS. They were washed with PBS containing 0.1% Tween 20, incubated for 45 min at room temperature with primary antibody and then incubated with the second antibody coupled to horseradish peroxidase. Several antibodies were used for immunodetection on Western blot: in a first incubation, to test HSP70 recognition, we used the primary rabbit polyclonal antibody N1 and then a second anti-rabbit antibody. N1 antibody was raised against *P. waltl* 74 × 10^3 M_r HSP70-like protein, its epitope is a 16 aa sequence lying about 57 residues upstream of the protein C-terminus and was chosen from a polypeptidic sequence of an inducible form of *P. waltl* HSP70 [3]. The membrane was then stripped of antibody and reprobed in a second incubation to test myc-epitope-tag recognition using the primary mouse monoclonal mAb9E10 myc antibody (Santa-Cruz Biotechnology) and a second anti-mouse antibody. In both cases, identification of the antigen-antibody complex was performed by an ECL Western blotting detection kit (Amersham).

RESULTS

Effects of hsp70 or hsc70 Antisense Oligos on Targeted mRNA in Oocytes

Antisense strategy was performed using antisense oligos rather than antisense RNAs. Indeed, due to high similarity between the nucleotide sequence of inducible hsp70 and constitutive hsc70 [8], *hsp70* or *hsc70* mRNAs might be inactivated simultaneously by *hsp70* or *hsc70* antisense RNAs. In contrast, *hsp70* or *hsc70* mRNAs must be distinctly inactivated by antisense oligos specific for their nucleotide sequence. Several pairs of hsp/hsc70 antisense (hspAS1, hspAS2; hscAS1, hscAS2) and sense (hspS1, hspS2; hscS1, hscS2) oligos were used (see Materials and Methods; Fig. 1). They were complementary or identical to the corresponding hsp70 mRNA or hsc70 mRNA. The following controls were done: oocytes were injected with either hsp70 or hsc70 sense oligos; oocytes were injected with αV oligo unrelated to any heat-shock mRNA and corresponding to αV integrin (see Materials and Methods) or were injected with H_20. Since *P. waltl hsp70* and *hsc70* genes reveal 72.5% identity in their nucleotide sequence, the specific effects on *hsp70* or *hsc70* mRNA induced by corresponding antisense oligos were analyzed at the RNA level by RT-PCR assays rather than Northern blot. Indeed, signals detected by Northern blot could not be attributed precisely to the *hsp70* or *hsc70* gene. Specific effects of hsp70 antisense oligos on targeted *hsp70* mRNA were thus analyzed. As shown in Fig. 2a, with both of the antisense oligos injected (hspAS1 or hspAS2), a drop in the amount of *hsp70* mRNA was detected and reflected degradation of the targeted *hsp70* mRNA. In contrast, the amount of *hsp70* mRNA did not seem to be significantly affected in control experiments (C, hspS1, or hspS2). This degradation is sequence specific since it did not affect control *αV integrin* mRNA nor hsc70 mRNA (Figs. 2a and 4). In a similar manner, injection of hsc70 antisense oligos (hscAS1 or hscAS2) induced sequence-specific degradation of the targeted *hsc70* mRNA, whereas sense or αV oligos did not have significant effect (Figs. 3a and 4).

Quantitation of autoradiographs shown in Figs. 2b and 3b and obtained from five experiments demonstrated that hsp70 antisense oligos induced specific degradation of *hsp70* mRNA by 60 to 80%, and that

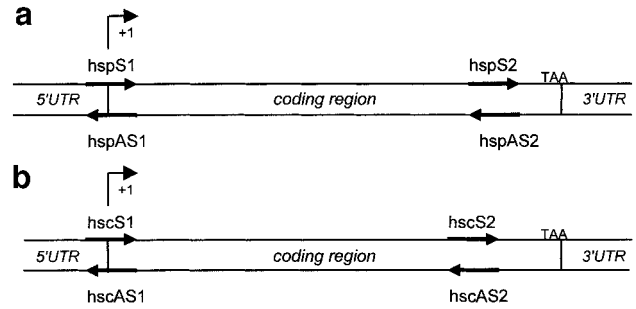

FIG. 1. Schematic representation of position and orientation of sense (hspS1, hspS2, hscS1, hscS2) and antisense (hspAS1, hspAS2, hscAS1, hscAS2) oligos on *hsp70* cDNA (a) or *hsc70* cDNA (b); +1, initiation site of transcription; TAA, stop codon; *UTR:* Un-Translated Region.

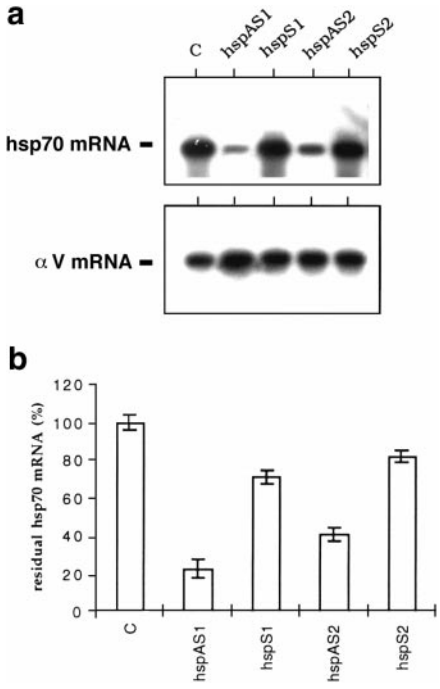

FIG. 2. Effects of hsp70 oligos on targeted *hsp70* mRNA by RT-PCR assays followed by Southern blot. (a) Autoradiograph resulting from Southern hybridization using ^{32}P-labeled hsp70 or αV probes. RT-PCR assays were performed using specific primers for the *hsp70* cDNA and the *αV* cDNA from oocytes 5 h after injection of either hsp70 antisense (hspAS1, hspAS2) or sense (hspS1, hspS2) oligos. C, oocytes injected with H2O. An equal amount of PCR product was loaded onto the gels. Note that the amount of *αV* mRNA was not affected after any injection. (b) Quantitation using the PhosphoImager of the hsp70 and αV PCR products detected by Southern hybridization and representing the mean value of five independent experiments. Results were standardized as a mean percentage of control (C). The quantitation of hybridization signals using hsp70 mRNA as probe is made with respect to the corresponding hybridization signals using αV mRNA considered as the standard for RT-PCR assays.

hsc70 antisense oligos induced specific degradation of *hsc70* mRNA by 80 to 90%, as compared with controls. This decrease was observed as early as 5 h after antisense oligo injection as well as 24 h after injection (data not shown).

Effects of hsp70 and hsc70 Antisense Oligos on Lampbrush Chromosome Structure

Lampbrush chromosomes from stage V–VI oocytes of *Pleurodeles* are highly distinctive and actively transcribed. They can be identified due to the presence of obvious landmark loops such as granular and globular, which show distinctive morphology related to the size and type of organization of their matrix components, and are observed at constant and reproducible sites along the chromosome axis [36] (Figs. 5a, a′).

For a limited period of 24 h after injection of either hsp70 antisense or hsc70 antisense, sense or αV oligos, the same effects were induced whatever the dose of oligos used (see Materials and Methods). Dramatic cytological effects on lampbrush chromosome structure started to appear within 10 min: all lateral loops of the lampbrush chromosomes progressively retracted into the chromosome axes, which reflected inhibition of transcription. Five hours after injection, lampbrush chromosomes were devoid of all normal loops, and only granular and globular loops were observed (Figs. 5b, b′). These effects were similar to those which occurred when oocytes of *P. waltl* were incubated with actinomycin D or α-amanitin [21], or when oocytes were microinjected with anti-HSP/HSC70 antibody [10].

Beyond 24 h after injection, the effects induced by hsp70 antisense oligos were different from those induced by hsc70 antisense, hsc70 sense, αV oligos or H2O. Twenty-four hours after injection of any hsp70 antisense oligo, all bivalents exhibited the feature of metaphasic chromosomes: the chromosomal axes were

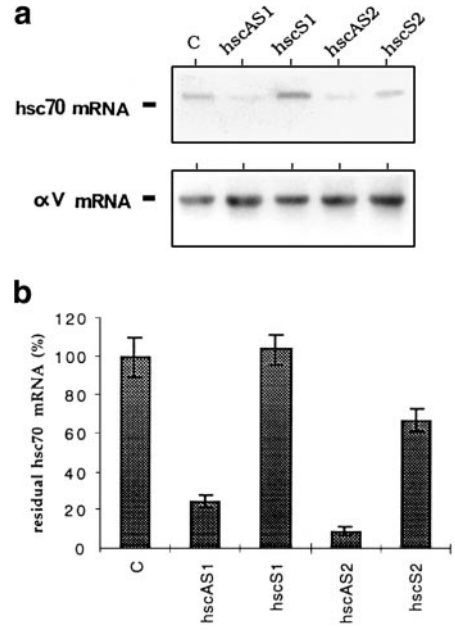

FIG. 3. Effects of hsc70 oligos on targeted *hsc70* mRNA by RT-PCR assays followed by Southern blot. (a) Autoradiograph resulting from Southern hybridization using ^{32}P-labeled hsc70 or αV probes. RT-PCR assays were performed using specific primers for the *hsc70* cDNA and the *αV* cDNA from oocytes 5 h after injection of either hsc70 antisense (hscAS1, hscAS2) or sense (hscS1, hscS2) oligos. C, oocytes injected with H2O. An equal amount of PCR product was loaded onto the gels. Note that the amount of *αV* mRNA was not affected after any injection. (b) Quantitation using the PhosphoImager of the hsp70 and αV PCR products detected by Southern hybridization and representing the mean value of five independent experiments. Results were standardized as a mean percentage of control (C). The quantitation of hybridization signals using hsc70 mRNA as probe is made with respect to the corresponding hybridization signals using αV mRNA considered as the standard for RT-PCR assays.

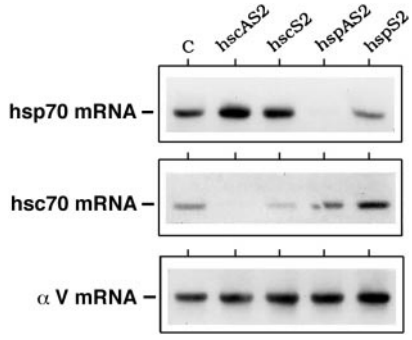

FIG. 4. Specifity of oligos on targeted mRNA degradation by RT-PCR assays followed by Southern blot. Autoradiograph resulting from Southern hybridization using ^{32}P-labeled hsp70, hsc70, or αVprobes. RT-PCR assays were performed using specific primers for the hsp70 cDNA (hsp70 mRNA), the hsc70 cDNA (hsc70 mRNA), and the αVcDNA (αVmRNA) from oocytes 5 h after injection of either hsc70 antisense (hscAS2) and sense (hscS2) oligos or hsp70 antisense (hsp70 antisense) and sense (hsp70 sense) oligos. C, control oocytes injected with H_2O. An equal amount of PCR products was loaded onto the gels. Note that the amount of αVmRNA was not affected after any injection.

extremely condensed and foreshortened (Fig. 5c). Such modifications were observed 48 h after injection (Fig. 5d) and even 6 days later. Thus, modifications induced by hsp70 antisense oligos were not reversible. In contrast, beyond 24 h after several hsc70 antisense or control oligo injections, a recovery process was observed: 24 h after injection, chromosomes began to lengthen and exhibited detectable loops (Fig. 5c′). By 24–48 h, lampbrush chromosomes completely regained their normal morphology: lateral loops were as developed as in noninjected oocytes, and chromosomal axes were completely reextended (Fig. 5d′). The reversible inhibition of transcription induced by injection of any oligo was previously observed in amphibian oocytes at the level of lampbrush chromosome loops by Tsvetkov *et al.* [18], and was considered as non-sequence specific.

Thus, while hsp70 antisense oligos led to irreversible inhibition of transcription, hsc70 antisense oligos, like other oligos, induced a reversible inhibition of transcription.

Effects of hsp70 and hsc70 Antisense Oligos on Nucleolar Transcription

In order to elucidate whether HSP70 and/or HSC70 were involved in nucleolar transcription, we carried out autoradiography on nuclear spreads of oocytes injected 24 h earlier with hsp70 or hsc70 antisense oligos and then incubated in culture medium containing [^{3}H]uridine for 24 h (see Materials and Methods). *P. waltl* oocyte nucleoli are extrachromosomal and can be easily distinguished from lampbrush chromosomes. Beyond 24 h after hsc70 antisense oligo injection, lateral loops of chromosomes and nucleoli appeared to be strongly labeled (Figs. 6a, a′) as observed in control oocytes. Beyond 24 h after hsp70 antisense oligo injection, while chromosomal transcription was arrested, nucleolar transcription was not. Autoradiographs exhibited strongly labeled nucleoli (Figs. 6b,b′ and c,c′). Thus, hsc70 or hsp70 antisense oligos do not affect nucleolar transcription. These results suggested that neither HSC70 nor HSP70 are involved in nucleolar transcriptional processes.

Effects of hsp70 Antisense Oligos on HSP/HSC 70 Syntheses

To check whether the irreversible inhibition of transcription of lampbrush chromosomes induced by hsp70 antisense oligos was associated with a change in the amount of neosynthesized HSP70 in the germinal vesicle (gv), proteins were extracted from the gvs of noninjected oocytes or injected 24 h earlier and then incubated with [^{35}S]methionine (cf. Materials and Methods). Only this time period was considered, since inhibition of transcription was shown to be induced by any injected oligo during the first 24 h after injection. Two-dimensional gel separations of proteins were carried out and gels were processed for autoradiography. The position of HSP/HSC70 proteins was determined using anti-HSP/HSC70 antibody (N1) which recognized three polypeptides of the HSP/HSC70 family in *Pleurodeles* oocytes [9]. Such immunodetected protein pattern was obtained in injected oocytes whatever the oligo used (Fig. 7I). The pattern of newly synthesized proteins in hsp70 sense injected oocytes was the same as in αV oligo-injected oocytes or in noninjected oocytes. It shows three distinctive spots corresponding to neosynthesized HSP/HSC70 proteins (Fig. 7S). In contrast, in hsp70 antisense oligo-injected oocytes, significant changes were observed. Among these three spots, two were shown to be specifically affected and corresponded to the basic forms (Fig. 7AS). These data therefore suggested that hsp70 antisense oligo injection does not affect the stored HSP/HSC70 proteins but reduced the synthesis of two of their several forms. These two forms could be supposed to correspond to HSP70.

Recovery Experiments

In order to determine whether chromosomal transcription may be recovered after hsp70 antisense oligo injection, the neosynthesis of HSP70 was restored by injection of synthetic *hsp70* mRNA (see Materials and Methods) and transcription was then analyzed at the level of lampbrush chromosome structure.

Two types of synthetic mRNA were used: capped, sense-stranded transcripts were synthesized from both the wild-type *hsp70* clone and the mutant myc-tagged

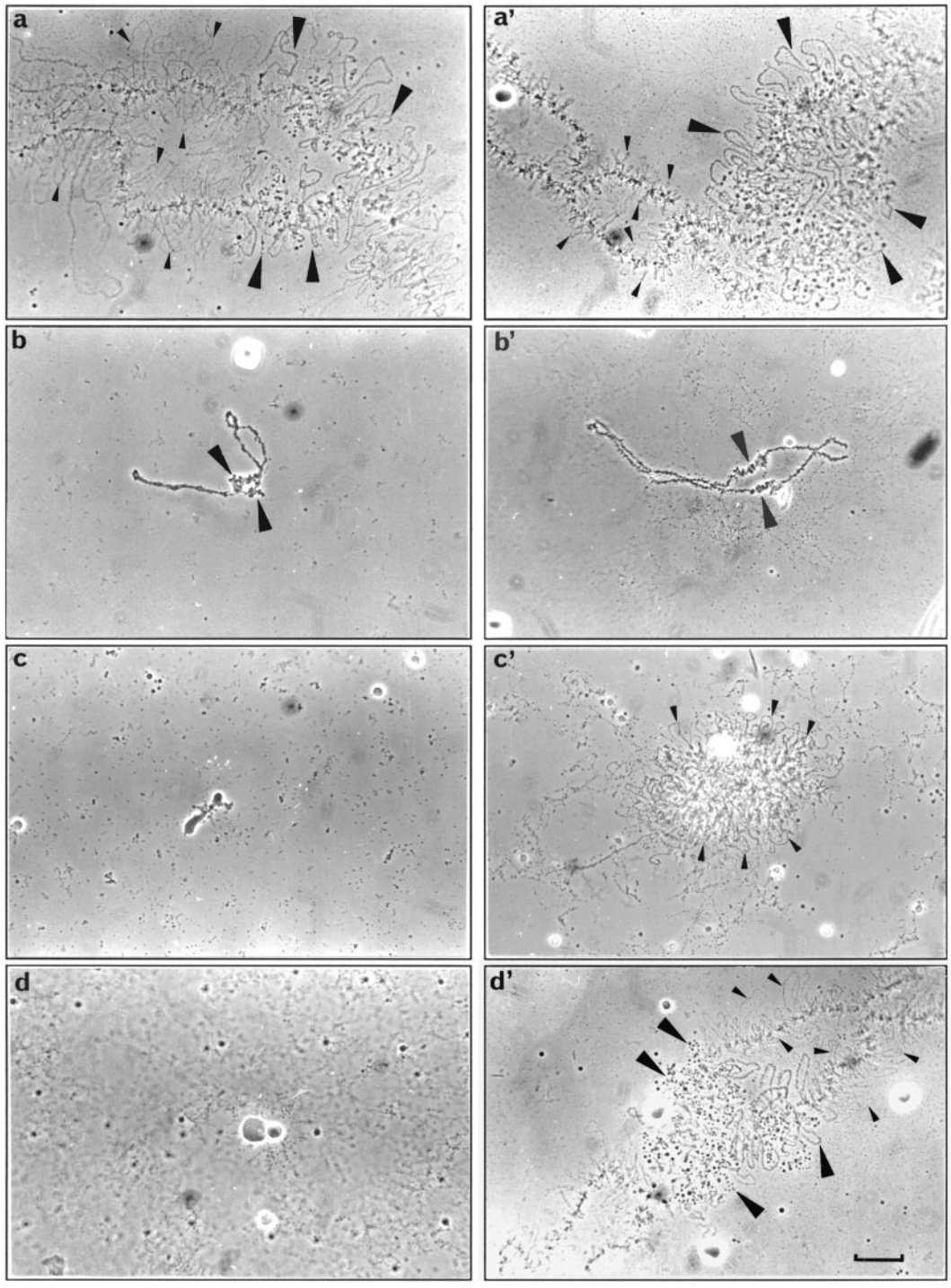

FIG. 5. Progressive modifications in lampbrush chromosome structure after injection of oligos, as seen by phase-contrast microscopy at the level of one defined bivalent, bivalent VII. (a, a′) Noninjected oocyte. The bivalent VII exhibits normal loops (small arrowhead) and a bundle of globular (large arrowhead) and granular loops which allows its identification. (b, c, d) Irreversible effects on lampbrush chromosome structure after injection of hsp70 antisense oligos. (b) At 5 h after injection, normal loops are retracted but typical globular loops (arrowhead) are maintained; the chromosomal axis is shortened. (c) At 24 h after injection, all lateral loops have disappeared and the chromosomal axis is strongly condensed. (d) The bivalent exhibits metaphasic chromosome features even 48 h after injection. (b′, c′, d′) Revesible effects on lampbrush chromosome structure after injection of hsc70 antisense oligos. The same effects were observed after injection of control oligos (hspS1, hspS2, hscS1, hscS2, or an unrelated oligo). (b′) At 5 h after injection, normal loops are retracted, but typical globular loops (arrowhead) are maintained, and the chromosomal axis is shortened. (c′) At 24 h after injection, lateral loops progressively redevelop and the chromosomal axis begins to reextend. (d′) At 48 h after injection, normal chromosome morphology is recovered. Bar represents 10 μm.

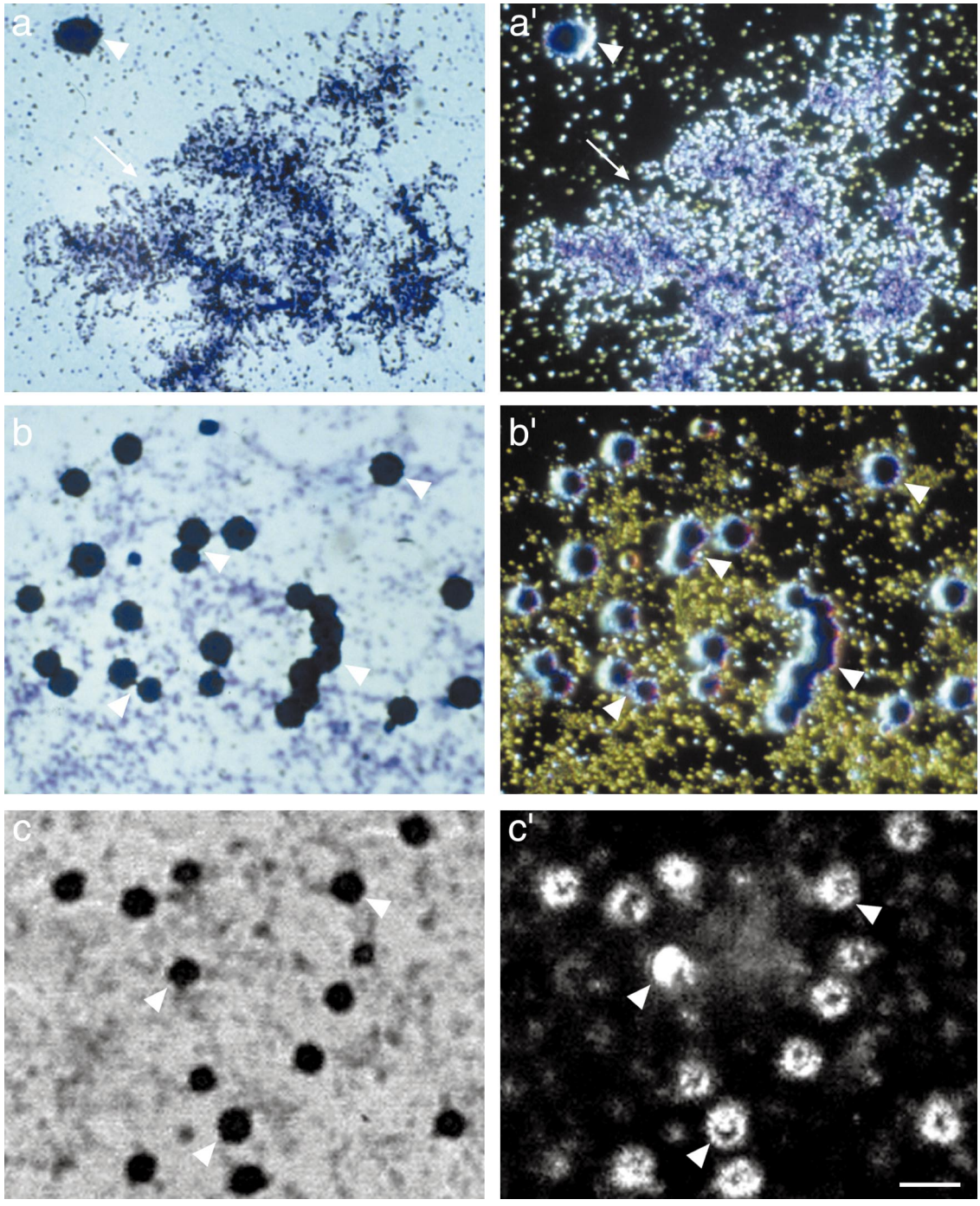

FIG. 6. Effects of hsp70 and hsc70 antisense oligos on nucleolar transcription, as seen by autoradiography. (a, a′) at 24 h after hsc70 antisense oligo injection nucleoli (arrowheads) and lampbrush chromosomes loops (arrows) were strongly labeled as seen in a bright-field (a) or with dark background (a′) illumination. Note that the numerous free particles which correspond to RNP particles were also strongly labeled. Same results were obtained after injection of hspS1, hspS2, hscS1, hscS2, or αV oligo. (b, b′) at 24 h after hsp70 antisense oligo injection, while lampbrush chromosomes were retracted, nucleoli (arrowheads) were strongly labeled as seen in a bright-field (b) or with dark background (b′) illumination; (c, c′) 24 h after hsp70 antisense oligo injection. Analysis of labeled nucleoli (arrowheads) by laser scanning confocal microscopy in contraste phase (c) or by reflection (c′). The reflected image clearly showed the labeling over the nucleoli. Bar represents 5 μm.

hsp70 clone. Indeed, a mutant HSP70 protein was necessary to follow the kinetics of injected mRNA, because endogenous HSP70 is stored in large amounts in the oocyte. The corresponding *in vitro* translation products were analyzed by autoradiography or were assayed on immunoblots with both primary antibodies: N1, which

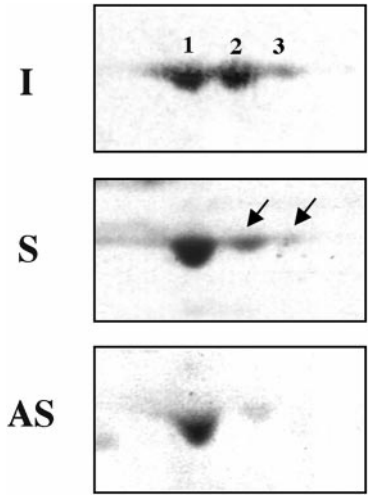

FIG. 7. Autoradiographs of two-dimensional polyacrylamide gels and HSP/HSC70 immunolocalization. Magnification of HSP/HSC70 region. (I) HSP/HSC70 immunolocalization. N1 antibody recognized three forms of HSP/HSC70 in the germinal vesicle of noninjected oocytes. The same result was obtained with either hsp70 sense or antisense-injected oocytes. (S) Autoradiographs of newly synthesized proteins from hspS2 sense oligo-injected oocytes. Three spots were detected. The same result was obtained with hspS1 sense or αV oligo injection. (AS) Autoradiographs of newly synthesized proteins from hspAS2 antisense oligo-injected oocytes. The intensity of two (arrows) of the three spots detected in (S) was reduced. The same result was obtained with hspAS1 antisense oligo.

is specific for the *P. waltl* HSP/HSC70 proteins [9], and mAb 9E10, which is specific for the myc-epitope-tag [37]. Autoradiograms provided evidence for two major bands of 70 and 80 kDa corresponding to the syntheses of wild-type HSP70 and the fusion myc-tagged HSP70, respectively (Fig. 8). The molecular weight of 80 kDa is

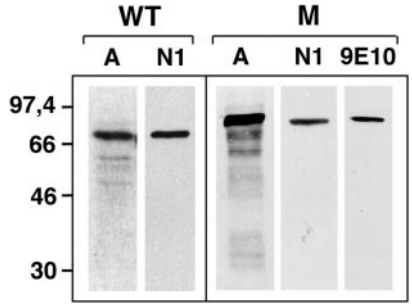

FIG. 8. *In vitro* translation of wild-type (WT) and mutant myc-tagged HSP70 (M) proteins. ^{35}S-translated proteins were mono electrophoresed and were processed for either autoradiography (A) or immunodetection (N1 and 9E10). Blots were incubated either with N1 antibody (N1) specific for the *Pleurodeles waltl* HSP/HSC70 proteins or with 9E10 antibody (9E10) specific for the myc-epitope-tag. Immunodetection was revealed by chemiluminscence. Molecular mass markers are in Daltons $\times 10^{-3}$. A band of 70 kDa was recognized by N1 in WT and another of 80 kDa was recognized by 9E10 in M.

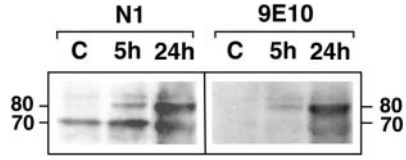

FIG. 9. *In vivo* translation of myc-tagged HSP70 fusion protein 5 and 24 h after mutant myc-tagged hsp70 mRNA injection. The oocytes were injected with hsp70 antisense oligo. Twenty-four hours later they were injected again with mutant myc-tagged hsp70 mRNA. Total proteins were subsequently extracted 5 or 24 h later. (C) Total proteins extracted from oocytes injected only with hsp70 antisense oligo. Corresponding Western blots were incubated with N1 antibody (N1), stripped of bound antibodies, and then reprobed with 9E10 anibody (9E10). N1 antibody recognized one band of 70 kDa in (C), two bands of 70 and 80 kDa at 5 and 24 h in the injected oocytes. Using 9E10 antibody, no band was detected in control oocytes while a band of 80 kDa was detected 5 and 24 h in the injected oocytes.

consistent with the fact that translation of this fusion protein begins in the 6 myc-epitope region (10.3 kDa). As expected, the 70 kDa is only recognized by N1 antibody while the 80-kDa fusion protein is recognized by both N1 and 9E10 antibodies.

The kinetics of exogenous HSP70 *in vivo* translation was carried out using Myc-tagged *hsp70* mRNA. It was then followed from 5 to 24 h after synthetic mRNA injection. Oocytes were isolated and their total proteins assayed on immunoblots with the same antibodies as those used for *in vitro* assays. While N1 recognized both endogenous stored HSP70 (band of 70 kDa) and exogenous myc-epitope-tagged HSP70 (band of 80 kDa), a unique fusion protein with a molecular weight of 80 kDa was recognized by both N1 and 9E10 antibodies in the whole oocyte (Fig. 9). It was already detected at 5 h. To check that the exogenous protein did migrate into the nucleus as did the endogenous one, nuclear and cytoplasmic oocyte proteins were assayed on immunoblots with N1 and 9E10 antibodies. Results showed that exogenous myc-epitope-tagged HSP70, as well as endogenous stored HSP70, were present in both cytoplasmic and nuclear compartments (Fig. 10). These results provided evidence for the *in vivo* restoration of HSP70 neosynthesis and its nuclear transfer.

To determine whether recovery of HSP70 protein synthesis was able to induce chromosomal decondensation, wild-type or mutant *hsp70* synthetic mRNAs were injected in the oocytes where transcription was previously inhibited by hsp70 antisense oligos. Lampbrush chromosomes from such oocytes did not regain their normal morphology: they remained extremely condensed and foreshortened, exhibiting a metaphasic aspect. Transcription was not restored (Fig. 11). These results suggested that the restoration of HSP70 synthesis is not sufficient to induce decondensation of lampbrush chromosomes.

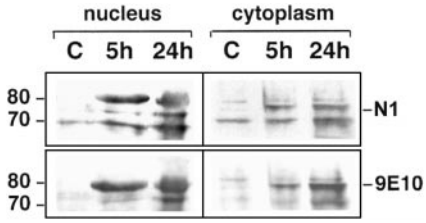

FIG. 10. Cellular localization of the newly synthesized myc-tagged HSP70 protein. The oocytes were injected with hsp70 antisense oligo; 24 h later they were injected again with mutant myc-tagged hsp70 mRNA. Nuclear and cytoplasmic compartments were subsequently extracted 5 and 24 h later. Corresponding Western blots were incubated with N1 antibody (N1), stripped of bound antibodies, and then reprobed with 9E10 anibody (9E10). N1 antibody recognized one band of 70 kDa in (C), two bands of 70 and 80 kDa at 5 and 24 h in the injected oocytes, in both nuclear and cytoplasmic compartments. Using 9E10 antibody, no band was detected in control oocytes while a band of 80 kDa was detected 5 and 24 h in the injected oocytes, in both nuclear and cytoplasmic compartments.

In order to ascertain whether nonrecovery of transcription was not due to general sufferance of injected oocytes, we tested whether other cellular processes, such as progesterone-induced maturation, may be activated in oocytes injected with hsp70 antisense oligos. Twenty-four hours after injection of hsp70 antisense, hsc70 antisense, sense, or αV oligos, meiotic maturation of injected oocytes was checked after a 16-h incubation in progesterone-containing medium (see Materials and Methods). In oocytes injected with hsp70 or hsc70 antisense oligos, the typical "white spot" appeared, indicating that oocyte maturation was processing (data not shown). Such results demonstrated that inactivation of either hsp70 or hsc70 mRNA did not affect maturation processes.

DISCUSSION

The aim of this study was to assess the specific role of HSP70 or HSC70 proteins in transcriptional processes in the amphibian oocyte. Using an antisense strategy, we were able to show that only inducible HSP70 was involved in chromosomal decondensation related to transcriptional activity, while HSC70 was not.

An inhibition of transcription, reflected by lampbrush loop retraction and lampbrush chromosomal condensation, was induced by all oligos used. However, such inhibition was irreversible after injection of any hsp70 antisense oligo, even at low concentrations (12 ng), whereas it was reversible after hsc70 antisense oligos and all others. The reversible inhibition of transcription induced by different other oligos was previously observed in amphibian oocytes at the level of lampbrush loops [18]. According to these authors, it may have been due to polyanionic charges of oligos which would attract basic proteins or ions. Since these differential effects between hsp70 and hsc70 oligos were observed not only for the two pairs presented here but also for numerous others we used (data not shown), these effects could not be attributed to a differential stability between the corresponding oligos. We also demonstrated that neither HSP70 nor HSC70 was involved in nucleolar transcriptional processes. Indeed, injection of hsp70 antisense or hsc70 antisense oligos did not interfere with nucleolar transcriptional processes, as shown by autoradiography. Thus, HSP70 was specifically involved in chromosome transcriptional processes. Such a role for HSP70 might be tissue specific, since this heat-inducible *hsp70* gene has been shown to be constitutively expressed during oogenesis [3]. It is noteworthy that even though HSC/HSP70 are

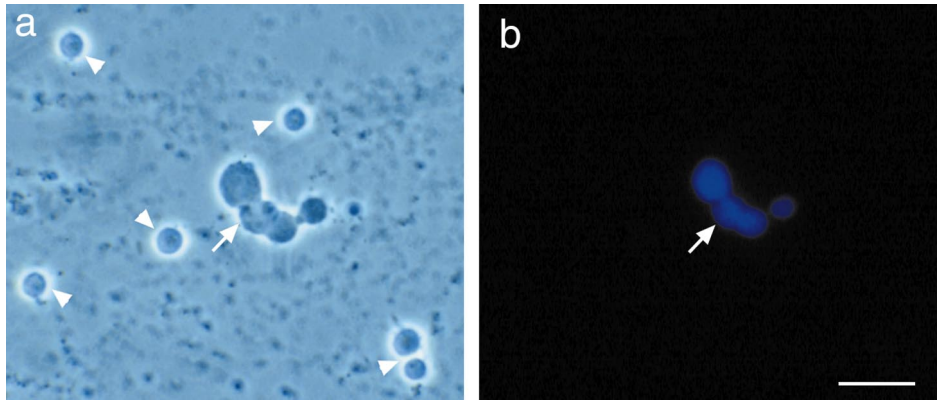

FIG. 11. Effect of restoration of HSP70 synthesis on chromatin decondensation. Wild-type hsp70 synthetic mRNA was injected in the oocytes where transcription was previously inhibited by hsp70 antisense oligos. Phase-contast (a) and fluorescence images (b) of lampbrush chromosomes stained with 4′,6-diamidino-2-phenylindole (DAPI). All bivalents exhibit the same features as this one (arrows). They are extremely condensed and foreshortened exhibiting a metaphasic aspect. Transcription was not restored. Arrowheads pointed to the nucleoli. Bar represents 7 μm.

known to share a common intracellular localization which could have "functional homology" [38], our study led us to suggest that in the oocyte, these two proteins would play different roles.

Analysis of protein synthesis led us to assume that only neosynthesized HSP70 was specifically involved in control of polymerase II transcriptional processes. Indeed, from our results, the irreversible inhibition of chromosomal transcription might be related to the drop in neosynthesis of HSP70. Furthermore, results from protein staining as well as immunoblotting showed that the drop in the neosynthesized fraction of HSP70 did not affect the total amount of stored HSP70. These results suggest that stored and newly synthesized HSP70 have different properties that may be due to posttranslational modifications, such as an association with other proteins. Different roles between a newly synthesized and a stored form were also reported for $p34^{cdc2}$ in the *Xenopus* egg [39].

These new data not only confirm but also extend those previously obtained [10], suggesting a role for HSP/HSC70 in the control of chromosomal transcription. Here we provide evidence for specific involvement of neosynthesized HSP70 in chromosomal decondensation related to transcriptional activity. When neosynthesized HSP70 was absent from the nucleus, initial swelling of polymerase II transcriptionally inactive chromosomes could not occur. Thus, neosynthesized HSP70 might play a direct or indirect role in chromatin modifications. Up to the present, no data have been reported about a direct association between HSP70 and chromatin. In contrast, an indirect role for HSP70 might be exerted by mediating the nuclear import of factors directly involved in chromosomal decondensation related to transcriptional activity. The presence of decondensation factors in oocyte nuclei was previously suggested by both *in vitro* [40–42] and *in vivo* experiments [43]. Indeed, when condensed sperm chromatin was injected into the nucleus of amphibian oocytes, it became actively transcribed exhibiting lampbrush structure [43].

Surprisingly, even when neosynthesis of HSP70 was restored by hsp70 mRNA injection and did migrate into the nucleus, transcription on lampbrush chromosome was not. We can assert that nonrecovery of transcriptional activity was not due to general sufferance of injected oocytes, since other cellular processes, such as nucleolar transcription and progesterone-induced GVBD, were not affected. Results of recovery experiments suggest that neosynthesized HSP70 was necessary but not sufficient to ensure resumption polymerase II transcription.

In conclusion, our results suggest a role for neosynthesized HSP70 in chromosomal transcription activity, most likely through its association with other proteins involved in chromosomal decondensation.

The present work was supported by the CNRS, Université Pierre et Marie Curie, Association pour la Recherche contre le Cancer (ARC 6309) and grants from Ministère de la Recherche (ACC-SV 4). C.D.-F. was supported by a fellowship from Ministère de l'Education Scientifique et de la Recherche (MESR). The plasmid PRN3 was kindly provided by Dr. Patrick Lemaire. We are grateful to Dr. Chandra Pyne for going through the English version. We thank Ms. M. Barre and Mr. G. Géraud (Institut Jacques Monod), Mr. J. Desrosier (UMR 7622), and Mr. P. Nguyen (C.T.P.I, UFR 927) for the artwork.

REFERENCES

1. Nover, L., and Scharf, K. D. (1997). Heat stress proteins and transcription factors. *Cell. Mol. Life Sci.* **53,** 80–103.
2. Srinivas, K., and Swamynathan, S. K. (1996). Role of heat-shock transcription factors in stress response and during development. *J. Biosci.* **21,** 103–121.
3. Billoud, B., Rodriguez-Martin, M. L., Berard, L., Moreau, N., and Angelier, N. (1993). Constitutive expression of a somatic heat-inducible hsp70 gene during amphibian oogenesis. *Development* **119,** 921–932.
4. Herberts, C., Moreau, N., and Angelier, N. (1993). Immunolocalization of hsp70-related proteins constitutively expressed during *Xenopus laevis* oogenesis and embryogenesis. *Int. J. Dev. Biol.* **37,** 397–406.
5. Ali, A., Salter-Cid, L., Flajnik, M. J., and Heikkila, J. J. (1996). Isolation and characterisation of a cDNA encoding a Xenopus 70-kDa heat-shock cognate protein HSC70-I. *Comp. Biochem. Physiol.* **113,** 661–687.
6. Ali, A., Salter-Cid, L., Flajnik, M. J., and Heikkila, J. J. (1996). Molecular cloning of a cDNA encoding a *Xenopus laevis* 70-kDa heat-shock cognate protein HSC70-II. *Bioch. Biophys. Acta* **1309,** 174–178.
7. Angelier, N., Moreau, N., Rodriguez-Martin, M. L., Penrad-Mobayed, M., and Prudhomme, C. (1996). Does the chaperone heat-shock protein HSP70 play a role in control of developmental processes. *Int. J. Dev. Biol.* **40,** 521–529.
8. Delelis-Fanien, C., Penrad-Mobayed, M., and Angelier, N. (1997). Molecular cloning of a cDNA encoding the amphibian *Pleurodeles waltl* 70-kDa heat-shock cognate protein. *Biochem. Biophys. Res. Commun.* **238,** 159–164.
9. Prudhomme, C., Moreau, N., and Angelier, N. (1997). Conditions for a heat-shock response during oogenesis and embryogenesis of the amphibian *Pleurodeles waltl. Dev. Growth Differen.* **39,** 477–484.
10. Moreau, N., Lainé, M. C., Billoud, B., and Angelier, N. (1994). Transcription of amphibian lampbrush chromosomes is disturbed by microinjection of HSP70 monoclonal antibodies. *Exp. Cell Res.* **211,** 108–114.
11. Akagi, H., Patton, D. E., and Miledi, R. (1989). Discrimination of heterogenous mRNAs encoding strychnine-sensitive glycine receptors in Xenopus oocytes by antisense oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA* **86,** 8103–8107.
12. Colman, A. (1990). Antisense strategies in cell and developmental biology. *J. Cell. Sci.* **97,** 399–409.
13. Dagle, M. J., Walder, J. A., and Weeks, D. L. (1990). Targeted degradation of mRNA in Xenopus oocytes and embryos directed by modified oligonucleotides: Studies of An2 and cyclin in embryogenesis. *Nucleic Acids Res.* **18,** 4751–4757.
14. Prives, C., and Foukal, D. (1991). Use of oligonucleotides for antisense experiments in Xenopus laevis oocytes. *Methods Cell. Biol.* **36,** 186–210.

15. Pan, Z. Q., and Prives, C. (1988). Assembly of functional U1 and U2 human-amphibian hybrid snRNPs in Xenopus laevis oocytes. *Science* **241,** 1328–1331.
16. Pan, Z. Q., and Prives, C. (1989). U2 snRNA sequences that bind U2-specific proteins are dispensable for the function of U2 snRNP in splicing. *Genes Dev.* **3,** 1887–1898.
17. Hamm, J., Dathan, N. A., and Mattaj, I. W. (1989). Functional analysis of mutant *Xenopus* U2 snRNAs. *Cell* **59,** 159–169.
18. Tsvetkov, A., Jantsch, M., Wu, Z., Murphy, C., and Gall, J. G. (1992). Transcription on lampbrush chromosomes loops in the absence of U2 snRNA. *Mol. Biol. Cell.* **3,** 249–261.
19. Bona, M., Scheer, U., and Bautz, E. K. F. (1981). Antibodies to RNA polymerase II "b" inhibit transcription in lampbrush chromosomes after microinjection into living amphibian oocytes. *J. Mol. Biol.* **151,** 81–99.
20. Callan, H. G. (1986). "Lampbrush Chromosomes," Springer-Verlag, Berlin.
21. Penrad-Mobayed, M., Bonnanfant-Jaïs, M. L., N'Da, E., and Angelier, N. (1986). Evidence for a particular mode of transcription in globular loops of lampbrush chromosomes of the newt *Pleurodeles waltlii*. *Chromosoma* **94,** 319–328.
22. Marko, J. F., and Siggia, E. D. (1997). Polymer model of meiotic and mitotic chromosomes. *Mol. Biol. Cell* **8,** 2217–2231.
23. Lemaire, P., Garett, N., and Gurdon, J. B. (1995). Expression cloning of Siamois, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **7,** 85–94.
24. Rupp, R. A., Snider, L., and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8,** 1311–1323.
25. Turner, D. L., and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8,** 1434–1447.
26. Alfandari, D., Whittaker, C. A., De Simone, D. W., and Darribère, T. (1995). Integrin alphaV subunit is expressed on mesodermal cell surfaces during amphibian gastrulation. *Dev. Biol.* **170,** 249–261.
27. Moreau, N., and Boucher, D. (1981). Une méthode rapide d'extraction en masse des noyaux d'ovocytes de Pleurodèle (Amphibien, Urodèle). *Biol. Cell.* **42,** 185–188.
28. Wallace, R. A., Ho, T., Salter, D. W., and Jared, D. W. (1973). Protein incorporation by isolated amphibian oocytes: The role of follicular cells and calcium during protein uptake. *Exp. Cell Res.* **82,** 287–295.
29. Bonnanfant-Jaïs, M. L., and Mentré, P. (1983). Study of oogenesis in the newt *Pleurodeles waltlii* M. I.: "Ultrastructural study of the different stages of oocyte development. *J. Submicrosc. Cytol.* **15,** 453–478.
30. Gall, J. G. (1954). Lampbrush chromosomes from oocyte nuclei of the newt. *J. Morphol.* **94,** 283–351.
31. Angelier, N., Bonnanfant-Jaïs, M. L., Moreau, N., Gounon, P., and Lavaud, A. (1986). DNA methylation and RNA transcriptional activity in amphibian lampbrush chromosomes. *Chromosoma* **94,** 169–182.
32. Gall, J. G., Stephenson, E. C., Erba, H. P., Diaz, M. O., and Barsacchi-Pilone, G. (1981). Histone genes are located at the sphere loci of newt lampbrush chromosomes. *Chromosoma* **84,** 159–171.
33. O'Farell, H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250,** 4007–4021.
34. Laemmli, K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
35. Towbin, H., Stackelin, T., and Gordon, T. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
36. Lacroix, J. C. (1968). Etude descriptive des chromosomes en écouvillon dans le genre *Pleurodeles* (Amphibien, Urodèle). *Ann. Embryol. Morphogen.* **4,** 383–396.
37. Evan, G. I., Levis, G. K., Ramsay, G., and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* **5,** 3610–3616.
38. Boorstein, W. R., Ziegelhoffer, T., and Craig, E. A. (1994). Molecular evolution of the HSP70 multigene family. *J. Mol. Evol.* **38,** 1–17.
39. Chevalier, S., Tassan, J. P., Cox, R., Philippe, M., and Ford, C. (1995). Both cdc2 and cdk2 promote S phase initiation in *Xenopus* egg extract. *J. Cell. Sci.* **108,** 1831–1841.
40. Scmidt-Zachman, M. S., Hugle-Dorr, B., and Franke, W. W. (1987). A constitutive nucleolar protein identified as a member of the nucleoplasmin family. *EMBO J.* **6,** 1881–1890.
41. Philpott, A., Leno, G. H., and Laskey, R. A. (1991). Sperm decondensation in Xenopus egg cytoplasm is mediated by nucleoplasmin. *Cell* **65,** 569–578.
42. Leno, G. H., Mills, A. D., Philpott, A., and Laskey, R. A. (1996). Hyperphosphorylation of nucleoplasmin facilitates Xenopus sperm decondensation at fertilization. *J. Biol. Chem.* **271,** 7253–7256.
43. Gall, J. G., and Murphy, C. (1998). Assembly of lampbrush chromosomes from sperm chromatin. *Mol. Biol. Cell.* **9,** 733–747.

Received November 26, 1999
Revised version received July 13, 2000