Journal of Cellular Biochemistry 63:51–60 (1996)

Characterization of a New High-Temperature-Induced 66-kDa Heat-Shock Protein, Antigenically Related to Heat-Shock Protein 72

Andrea Delpino, Anna Maria Mileo, Vincenza Lapenta, Pierluca Piselli, Alessandra Verdina, and Lorenzo Polenzani

Laboratories of Biophysics (A.D., A.M.M., V.L., P.P., L.P.) and of Biochemistry (A.V.), Regina Elena Institute for Cancer Research, Center for Experimental Research, Via delle Messi d'Oro 156, 00158, Rome, Italy

Abstract M-14 human melanoma cells, following severe hyperthermic exposures, synthesized a heat-shock protein of 66 kDa (hsp 66), in addition to the major "classic" heat-shock proteins. This hsp 66 was not expressed following mild hyperthermic exposures sufficient to trigger the synthesis of the other heat-shock proteins. The induction of hsp 66 was observed also in Li human glioma cells treated at 45°C for 20 min. By contrast, hsp 66 was not induced in seven other human cell lines (both melanoma and nonmelanoma) when they were subjected to the same hyperthermic treatment. Immunological recognition experiments showed that hsp 66 cross-reacted with the inducible hsp 72, but not with the constitutive hsp 73. The possibility that hsp 66 is a breakdown product of hsp 72 was ruled out by the fact that Poly(A)$^+$ RNA extracted from cells treated at 45°C for 20 min was able to direct the synthesis of hsp 66 (together with hsp 72) in a message-dependent rabbit reticulocyte lysate, as well as in microinjected *Xenopus* oocytes. By contrast, only the hsp 72 was expressed using Poly(A)$^+$ RNA extracted from cells heated at 42°C for 1 h. Affinity chromatography experiments on ATP-agarose showed that hsp 66 did not bind ATP in vitro. hsp 66 was localized both in the cytoplasm (cytosol, mitochondria, and microsome fraction) and in the nuclei of cells recovered from a severe heat shock: this intracellular distribution closely corresponded to that of hsp 72. The nuclear-associated hsp 66 was found to be tightly bound to nuclear structures and could not be extracted by incubation in ATP-containing buffer.

Key words: hyperthermia, heat-shock proteins, melanoma cells, human cell lines, *Xenopus*

Among the mammalian heat shock protein (hsp) 70 family, four distinct proteins have been recognized and characterized in detail. According to the nomenclature used by Welch [1990], these are indicated as follows: (1) hsp 72, a protein expressed at very low levels under normal growth conditions, while it is induced to high levels after stress; (2) hsp 73, a constitutive, but also further inducible, protein termed also 73 kDa "cognate" protein (hsc 73); (3) GRP 78 (BIP) a protein resident in the lumen of the endoplasmic reticulum, typically induced by glucose starvation; and (4) P 75 (mt70 kDa hsp), a constitutive protein localized inside the mitochondrial matrix.

In some instances, proteins related to the hsp 70 family, but clearly distinct from the above-mentioned major components, have been described. For example, Konno et al. [1989] reported on a 67-kDa protein, antigenically related to hsps 73/72, localized at the cell surface of H-*ras*-transformed fibroblasts and able to elicit a strong antitumor response. Likewise, Van Buskirk et al. [1989] reported on a hsp 70-related protein, expressed on the plasma membrane of some antigen-presenting cells and functionally involved in the process of antigen presentation. More recently, Wadhwa et al. [1993], using mouse embryonic fibroblasts, characterized a 66-kDa protein (P 66/mot or mortalin), correlated with a limited life span (mortal phenotype). The gene coding for this protein was sequenced, and mortalin was identified as a previously unrecognized member of the hsp 70 family.

Received August 2, 1995; accepted April 14, 1996.

Address reprint requests to Dr. Andrea Delpino, Istituto Regina Elena per lo Studio e la Cura dei Tumori, Centro Ricerca Sperimentale, Laboratorio di Biofisica, Via delle Messi d'Oro 156 00158 Roma, Italy.

Lorenzo Polenzani's present address is Lab. Molecular Pharmacology, Angelini Ricerche, P.le della Stazione, 00040 S. Palomba-Pomezia, Roma, Italy.

In a previous paper [Delpino et al., 1992], we described a new hsp 70-related protein, the hsp 66, which was induced following severe heat shocks, together with the typical major hsps (i.e., hsp 90, hsp 73/72, and hsp 28). By contrast, following mild hyperthermic treatments, only these latter "classic" hsps were induced. In the experiments described in the present paper, this high-temperature-specific hsp 66 was further characterized.

MATERIALS AND METHODS

Cells, Growth Conditions, and Hyperthermic Treatments

The human melanoma cell line M-14 was used for most of the experiments reported in this paper. Four human melanoma cell lines originating from surgical specimens (Mont, JR, SB, and IR); four nonmelanoma human cell lines (one neuroblastoma, SK; one pancreatic adenocarcinoma, HPC-4; one glioma, Li and one histiocytic line, U-937) were also employed in some experiments. All these cell lines were cultured in RPMI 1640, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics (penicillin/streptomycin, 50 μg/ml each). Cells were routinely tested to be *Mycoplasma*-free, making use of the Hybricomb mycoplasma test kit (Biological Industries, Beth Haemek, Israel). Hyperthermic treatments were administered by submersion of sealed culture flasks in a thermoregulated water bath.

Radioactive Labeling of Cell Proteins, Preparation of Cell Lysates, and Electrophoretic Analysis

Labeling of cell proteins with [^{3}H]leucine (4–7 TBq/mMole) and cell lysis in detergent-containing buffer (Tris–HCl, 50 mM, pH 8; NaCl 150 mM; EDTA 5 mM; SDS 0.1% w/v; Na-DOC 0.5% w/v; NP-40 1% v/v; PMSF 1 mM, and aprotinin 20 μg/ml) were performed as previously described [Delpino et al., 1992]. Aliquots of cell lysates were mixed 1:1 with 2× Laemmli loading buffer, boiled, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [Laemmli, 1978], using 4% stacking and 10% resolving polyacrylamide gels. Autoradiographic detection of labelled proteins was performed according to Bonner and Laskey [1974].

Immunoprecipitation and Immunoblotting

For immunoprecipitation, aliquots of lysates from ^{3}H-labeled cells, containing about 5×10^6 cpm, were diluted to 300 μl with immunoprecipitation buffer (Tris–HCl, 10 mM, pH 7.6; NaCl 150 mM; EDTA 2 mM; Triton-X-100 1% v/v; SDS 0.1% w/v; PMSF 1 mM; aprotinin 20 μg/ml). After preclearance with normal mouse serum, samples were mixed with 10 μl of either monoclonal antibody (MAb) SPA 815 or MAb SPA 810 (both from StressGen, Victoria, Canada). Immunocomplexes were adsorbed onto protein G–protein A/agarose beads (Oncogene, Science Inc., Uniondale, NY); then, the immunoabsorbent was pelleted and washed several times with immunoprecipitation buffer, with high-salt immunoprecipitation buffer (as above, except NaCl was 0.5 M) and with Tris–HCl, 50 mM, pH 7.6. The immunoprecipitated material was finally solubilized in loading buffer and analyzed by SDS–PAGE.

For Western blot analysis of hsps expressed in M-14 cells subjected to different hyperthermic treatments aliquots of cell lysates were subjected to SDS–PAGE; proteins were then electrophoretically transferred from the polyacrylamide gel to a nitrocellulose membrane [Towbin et al., 1979]. Reactive sites on membrane were saturated in sequence with 0.2% gelatin and 2% nonfat dry milk; membrane was then probed with the MAb SPA 810 (1/1,000 dilution). Horseradish peroxidase-conjugated goat-anti mouse IgG (Bio-Rad Laboratories, Hercules, CA) was used as second antibody. Peroxidase activity on the membrane was revealed using 4-chloro-1-naphtol as a substrate.

Poly(A)$^+$ Extraction and Translation in Heterologous Systems

Poly(A$^+$) RNA from control and heat-treated cultures was extracted with the "Fast-track" kit (Invitrogen, San Diego, CA), following the instructions of the supplier; 1×10^8 cells yielded 10–30 μg of Poly(A)$^+$ RNA dissolved in RNase-free water (1 μg/μl) and stored at −80°C.

Samples of Poly(A)$^+$ RNA were translated in vitro in a message-dependent rabbit reticulocyte lysate system (Amersham Italia, Milano, Italy). Reaction mixtures containing 25 μl of lysate, 10 μCi of [^{3}H]leucine and 2 μg of the RNA to be tested were incubated for 1 h at 30°C. Translation products were analyzed by SDS–PAGE; aliquots of the translation cocktails were also

assayed by immunoprecipitation with the anti-hsp 72 MAb SPA 810.

Expression studies in *Xenopus laevis* oocytes were performed as described by Woodward et al [1992]. Briefly, follicle-enclosed *Xenopus* (Xenopus, CNRS, Montpellier, France) oocytes, at stages V and VI of development, were dissected from the ovary, maintained at 15°C in Barth's medium (NaCl 88 mM; KCl 1 mM; $Ca(NO_3)_2$ 0.33 mM; $MgSO_4$ 0.82 mM; $NaHCO_3$ 2.4 mM; HEPES 5 mM, pH 7.4) and microinjected with 75–100 ng of Poly(A)$^+$ RNA, dissolved in 50 nl of H_2O. Two days after injection, enveloping ovarian tissues were removed by collagenase treatment, and microinjected oocytes were labeled overnight in Barth's medium containing 100 μCi/ml of [^{3}H]leucine. After labeling, oocytes were washed several times in Barth's medium, then homogenized with a Potter-type homogenizer, manually operated. Samples were clarified by centrifugation and analyzed by SDS–PAGE; aliquots were also immunoprecipitated with the MAb SPA 810.

Affinity Chromatography on ATP–Agarose

Lysates from cultures heated at 45°C for 20 min were dialyzed against ATP buffer (HEPES 20 mM, pH 7.2; $MgCl_2$ 3 mM; KCl 20 mM; EDTA 0.1 mM; 2-mercaptoethanol 14 mM, and aprotinin 20 μg/ml), then adjusted to 20 mM $MgCl_2$ and applied to a 0.5-ml column of ATP-agarose (Sigma-Aldrich s.r.l., Milano, Italia), pre-equilibrated with ATP buffer. The column was washed with several volumes of high (0.5 M) KCl–ATP buffer then with regular ATP buffer. Finally, the bound material was eluted with 5 mM ATP. The ATP eluate was mixed with an equal volume of 2× loading buffer and analyzed by SDS–PAGE.

Subcellular Fractionation and Extraction of Nuclear Pellets

Subcellular fractionation was performed as described by Ohtsuka et al [1993] with only minor variations. Cells were harvested and disrupted with a Daunce glass homogenizer (15 strokes) in 5 vol of cold buffer (Tris–HCl, 20 mM, pH 7.6; EDTA 0.5 mM; sucrose 25 mM, and PMSF 1 mM). The homogenate was centrifuged at 700*g* for 10 min. The 700*g* pellet, including nuclei, unbroken cells, and cell debris, was resuspended in the same buffer, containing also 1% Triton-X-100, and briefly rehomogenized; nuclei were then purified by sedimentation through a 2 M sucrose pad (SW 50.1, 20,000 rpm for 1 h). The initial 700*g* supernatant was centrifuged at 7,000*g* for 10 min. The 7,000*g* pellet was saved as mitochondria fraction, while the 7,000*g* supernatant was further fractionated by centrifugation (60,000*g* for 1 h) into a 60,000*g* pellet (microsome fraction) and a supernatant (cytosol). Proteins present in each fraction were analyzed by SDS–PAGE.

Nuclear pellets were washed in extraction buffer (Tris–HCl, 50 mM, pH 7.6; KCl 25 mM; $CaCl_2$ 2 mM; $MgCl_2$ 4 mM; 2-mercaptoethanol 14 mM, and PMSF 1 mM) and resuspended in a small volume of the same buffer, containing 0 or 1 mM ATP. After 1-h incubation at room temperature, the nuclei were pelletted (1,000*g* for 5 min), and aliquots of both supernatants (nuclear extracts) and pellets (nuclear residual pellets) were analyzed by SDS–PAGE.

RESULTS

As shown in Figure 1, exposure of M-14 cells to mild heat stress (42°C for 1 h) was sufficient to strongly induce the hsp 72 and to slightly increase the expression of three other proteins (hsps 90, 73, and 28, respectively). Following a more severe hyperthermia (43.5°C, 40 min), the rate of synthesis of these "classic" hsps was enhanced and, in addition, a new protein at about 66 kDa was induced. The expression level of this 66-kDa protein was further increased when cells were subjected to more drastic hyperthermic treatments, such as 20 min at 45°C. These findings are consistent with our earlier observations describing the induction of a novel hsp, the hsp 66, in M-14 cells subjected to hyperthermic exposures strong enough to kill 50% or more of the whole cell population [Delpino et al., 1992].

It seemed of interest to ascertain whether the induction of the hsp 66 was peculiar to M-14 cells or whether this protein could be also induced in other cell lines. Therefore, the heat shock response elicited by a strong (45°C, 20 min) hyperthermic treatment was studied in four human melanoma cell lines (Mont, JR, SB, and IR, all established from surgical specimens) and in four nonmelanoma human cell lines (one neuroblastoma, SK; one pancreatic adenocarcinoma, HPC-4; one glioma, Li and one histiocytic line, U-937). Seven out of the eight cell lines tested were found to be negative for the induction of the hsp 66 (data not shown). However, in the glioma cell line Li, following exposure at

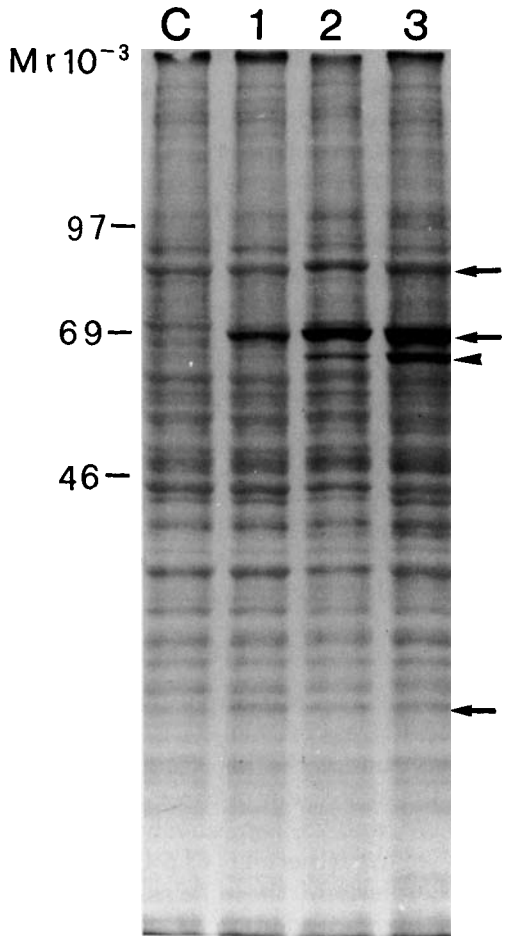

Fig. 1. SDS–PAGE analysis of proteins synthesized in M-14 cells following different hyperthermic regimens. M-14 cells were heated at 42°C for 1 h (*lane 1*), at 43.5°C for 40 min (*lane 2*), or at 45°C for 20 min (*lane 3*). *Lane C* (control), unstressed cells maintained at 37°C. Following the various hyperthermic treatments, cells were returned to 37°C and allowed to recover for 6 h. Labeling with [^{3}H]leucine was performed during the last 2 h of recovering. Aliquots of cell lysates, containing equal amounts of radioactivity, were loaded onto each gel lane. The positions of three [^{14}C]methylated protein markers (phosphorylase b, M_r = 97,000; serum albumin, M_r = 69,000 and ovalbumin, M_r = 46,000) are reported on the left side of the figure. *Arrows (right)*, major hsps (hsps 90, 73/72, and 28, respectively); *arrowhead,* hsp 66.

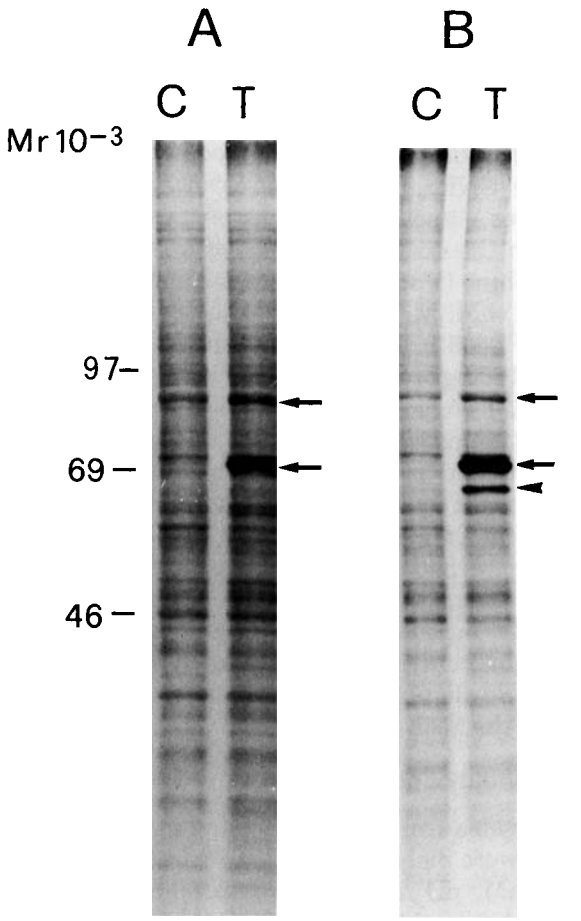

Fig. 2. SDS–PAGE analysis of proteins synthesized in Li cells following exposition at 42°C for 1 h **(A)** or at 45°C for 20 min **(B)**. Experimental conditions were as described in the legend of Fig. 1. *Lane C,* unstressed cultures; *lane T,* treated cultures. The position of molecular weight markers is reported on the left side. *Arrows (right)*, hsps 90 and 73/72; *arrowhead,* hsp 66.

45°C for 20 min, a strong induction of a 66-kDa protein was observed, in addition to the induction of hsp 90 and hsp 72. Like in M-14 cells, the 66-kDa protein was not induced when cells were heated at 42°C for 1 h (Fig. 2).

The antigenic relationship between the hsp 66 and hsps 72/73 was studied by immunoprecipitation experiments employing two MAbs: SPA 815, which specifically reacts with the constitutive hsp 73, and SPA 810, which recognizes only the inducible hsp 72.

The specificity of these MAbs was tested on a lysate obtained from cultures heated at 42°C for 1 h, in which both hsp 73 and hsp 72 were present, while hsp 66 was lacking. As shown in Figure 3, when this lysate was challenged with the MAb SPA 815 the hsp 73 was efficiently immunoprecipitated, along with limited amounts of the hsp 72, that weakly cross-reacted with this antibody (lane 2). On the other hand, the MAb SPA 810 was able to selectively immunoprecipitate the hsp 72 (lane 3). When these two MAbs were reacted with a lysate obtained from cultures heated at 45°C for 20 min (containing high amounts of hsp 66, in addition to hsps 73 and 72) it was found that the hsp 66 is immunoprecipitated, together with the hsp 72, by the MAb SPA 810 (lane 6), while it was not recognized by the MAb SPA 815, specific for the constitutive hsp 73 (lane 5).

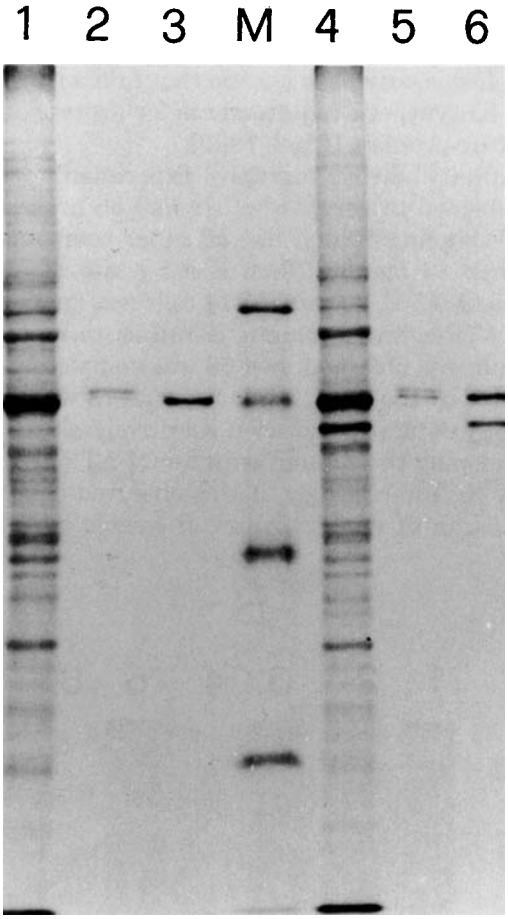

Fig. 3. Immunological recognition of hsp 66. Aliquots of labeled cell lysates obtained from cultures heated at 42°C for 1 h (*lanes 1,2,3*) or at 45°C for 20 min (*lanes 4,5,6*) were reacted with either the MAb SPA 815 (directed against the constitutive hsp 73) or the MAb SPA 810 (directed against the inducible hsp 72). Immunoprecipitated material was analyzed by SDS–PAGE and fluorography. *Lanes 1, 4,* starting cell lysate; *lanes 2, 5,* immunoprecipitation products with MAb SPA 815; *lanes 3, 6,* immunoprecipitation products with MAb SPA 810; *lane M,* displacement of four [^{14}C]methylated protein markers (phosphorylase b, M_r = 97,000; serum albumin, M_r = 69,000; ovalbumin, M_r = 46,000, and carbonic anydrase, M_r = 30,000).

Western blotting experiments were performed to exclude the possibility that the 66-kDa protein apparent (together with the hsp 72) in the immunoprecipitate obtained with the MAb SPA 810 could be only a coimmunoprecipitant, tightly bound to the hsp 72. Lysates from control cells and from cells heated at 42°C for 1 h, at 43.5°C for 40 min or at 45°C for 20 min were used in these experiments; the MAb SPA 810 was employed as primary antibody. The results of immunoblotting were in perfect agreement with the fluorographic patterns reported in Figure 1 and fully supported the immunoprecipitation data. In fact, as shown in Figure 4, a single, faint, band of 72 kDa was immunostained in lysates obtained from unheated cells (lane 1); the intensity of this band was greatly increased in lysates of cells heated at 42°C (lane 2), indicating a vigorous induction of this protein. By contrast, two proteins (72 and 66 kDa, respectively) were recognized by the MAb SPA 810 in lysates obtained from cells treated at 43.5° or 45°C (lanes 3 and 4). These two bands were also clearly detectable when the material immunoprecipitated with the MAb SPA 810 from a lysates of cells treated at 45°C (see lane 6 of Fig. 3) was blotted on nitrocellulose membrane and probed again with the same antibody (Fig. 4, lane 5).

Experiments were designed to distinguish whether the hsp 66 expressed in M-14 cells, subjected to severe hyperthermic treatments, was a degradation product (or a post-translational modification) of the hsp 72 or, alternatively, whether it was a distinct protein, coded by a different mRNA. For this purpose, Poly(A)$^+$ RNA preparations obtained from cultures subjected to mild (42°C, 1 h) or strong (45°C, 20 min) hyperthermic treatments were probed in a message-dependent reticulocyte lysate translation system.

It was found that in this in vitro assay, both preparations of Poly(A)$^+$ RNAs stimulated [^{3}H]leucine incorporation into polypeptides with

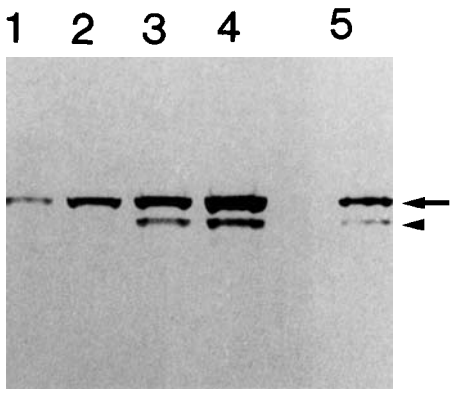

Fig. 4. Immunoblot analysis of hsps expressed in M-14 cells following different hyperthermic treatments. Lysates prepared from control cells (*lane 1*), from cells subjected to 42°C/1 h (*lane 2*), from cells subjected to 43.5°C/40 min (*lane 3*), and from cells subjected to 45°C/20 min (*lane 4*) were analyzed by Western blotting using the anti-heat-inducible hsp 72 MAb SPA 810. *Lane 5,* loaded with the material immunoprecipitated by the MAb SPA 810 from a lysate of cells treated at 45°C for 20 min (shown in Fig. 3, *lane 6*): this material was blotted on nitrocellulose membrane and reprobed with the same antibody. *Arrow (right)*, hsp 72; *arrowhead,* hsp 66.

roughly the same efficiency of Poly(A)$^+$ RNA isolated from unstressed cells. As shown in Figure 5A, the translation products of Poly(A)$^+$ RNAs isolated from control cells and from cells incubated at 42°C were similar, except for the presence of an additional band at about 72 kDa in the latter sample. This band corresponded to the hsp 72, as indicated by the fact that it was immunoprecipitated by the MAb SPA 810. By using as template the Poly(A)$^+$ RNA from cells treated at 45°C, two prominent polypeptides (72 and 66 kDa, respectively) were observed among the translation products. Both these polypeptides were recognized by the MAb SPA 810 and, therefore, they were identified as hsp 72 and hsp 66. As shown in Figure 5B, identical results were obtained when template activity of Poly(A)$^+$ RNAs isolated from cultures heated at 37°, 42°, and 45°C was assayed by microinjection on *Xenopus laevis* oocytes, a system that fulfils many of the biosynthetic requirements for expression of mature proteins [Bigel, 1990].

Affinity chromatography experiments were performed to verify whether hsp 66 possessed ATP-binding ability, like all other components of hsp 70 family. When a cell lysate of heat-treated (45°C, 20-min) M-14 cells was applied to an ATP-agarose column, a rather unexpected result was obtained: hsp 66 was completely excluded by the resin, while hsps 72/73 were efficiently retained and then selectively eluted by developing the column with 5 mM ATP (Fig. 6, lane 3). Interestingly, it was observed that the depletion of hsp 72 from cell lysates by ATP-

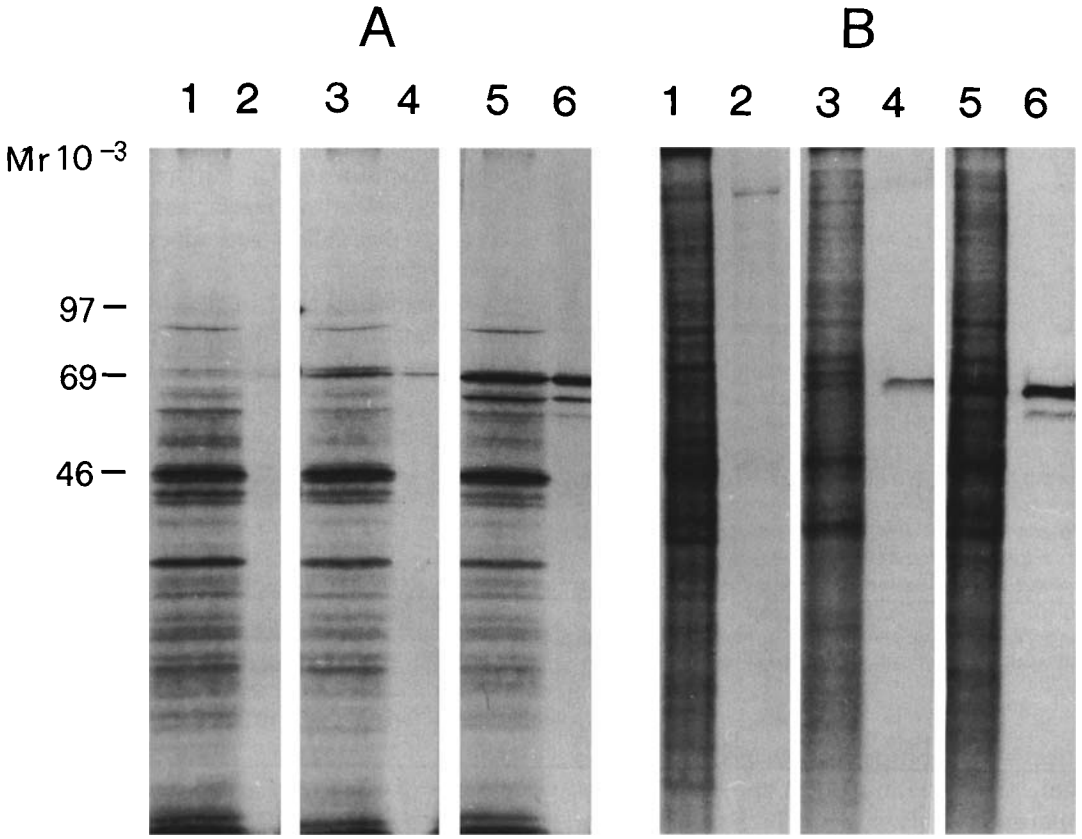

Fig. 5. SDS–PAGE analysis of translation products synthesized in rabbit reticulocyte lysates **(A)** and in *Xenopus* oocytes **(B)** programmed with Poly(A)$^+$ RNAs obtained from cultures subjected to mild or severe hyperthermic treatments. **A:** Reaction mixtures (25 μl) were programmed with 2 μg of Poly(A)$^+$ RNA from control cells (*lane 1*), from cells treated at 42°C for 1 h (*lane 3*), and from cells treated at 45°C for 20 min (*lane 5*). Translation products were analyzed by SDS–PAGE and fluorography; aliquots of each translation cocktail were also reacted with the anti-hsp 72 MAb SPA 810, and the immunoprecipitated material was electrophoresed aside the corresponding unfractionated sample (*lanes 2,4,6*). **B:** *Xenopus* oocytes were microinjected with 75–100 ng of Poly(A)$^+$ RNA from control cells (*lane 1*), from cells treated at 42°C for 1 h (*lane 3*), and from cells treated at 45°C for 20 min (*lane 5*). Oocytes were labeled with [^{3}H]leucine and translation products were analyzed by SDS–PAGE and fluorography. Aliquots of each homogenate were also reacted with the anti-hsp 72 MAb SPA 810, and the immunoprecipitated material was electrophoresed aside the corresponding unfractionated sample (*lanes 2,4,6*). The position of molecular-weight markers is reported on the left side.

agarose chromatography was not entirely efficient: in fact, a fraction of the labeled hsp 72 present in the starting lysate failed to bind ATP and was recovered in the flow-through fraction (Fig. 6, lane 2). To be sure that we were working with saturating amounts of resin, the unbound material was saved and re-applied to a new ATP-agarose column, but no further binding of the hsp 72 (nor of the hsp 66) was obtained (data not shown).

The intracellular localization of hsps 72 and 66 was studied using the cell fractionation protocol described in Materials and Methods. In these experiments, cultures were heated at 45°C for 20 min and allowed to recover for 18 h at 37°C. Labeling with [^{35}S]methionine was performed at 4 and 6 h post-treatment (when both the hsp 66 and the hsp 72 were synthesized at high rate); cultures were then chased for 12 h, to allow the newly synthesized proteins to distribute among the different intracellular compartments. As shown in Figure 7, the intracellular distribution of hsp 66 was closely coincident to that of hsp 72. Both these proteins were found to be localized both in the cytoplasm and in the nucleus; inside the cytoplasm, the most part of hsps 72 and 66 was recovered with the 60,000*g* supernatant (cytosol) but detectable amounts of them were also co-purified with mitochondria, as well as with microsome fraction (it should be noted that, on a per cell basis, the amount of material layered in lane 1 was about 10-fold less than that layered in the other lanes of the gel). The intracellular distribution of hsps 72 and 66 remained remarkably constant in cells that, following the initial heat shock and the pulse-chase procedure, were subjected to a second hyperthermic treatment (45°C for 10 min) and then immediately processed for subcellular fractionation. The only variation that could be observed was a small increase in the amount of the nuclear-associated hsp 72, while the intensity of the band corresponding to the nuclear-associated hsp 66 was unchanged (data not shown).

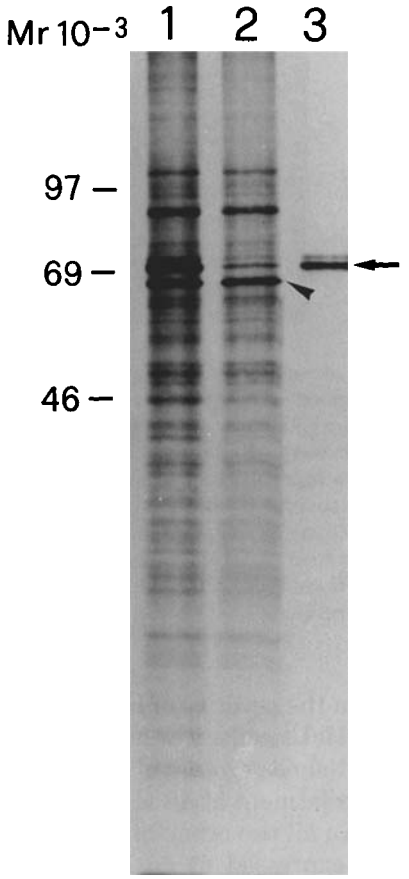

Fig. 6. Affinity chromatography on ATP–agarose. ^{3}H-leucine-labeled lysates from cultures treated at 45°C for 20 min were applied to a column of ATP–agarose. After exhaustive washing with high and low salt, the column was developed with 5 mM ATP. Samples from the starting lysate (*lane 1*), from the flowthrough material (*lane 2*), and from ATP-eluted material (*lane 3*) were analyzed by SDS–PAGE and fluorography. The position of molecular-weight markers is reported on the left side. *Arrow (right)*, hsps 73/72; *arrowhead*, hsp 66.

Further experiments were performed to determine whether the nuclear-associated hsps 72 and 66 were tightly or loosely bound to nuclear structures, and to test the ability of exogenous ATP to release them from isolated nuclei. For this purpose, purified nuclei were resuspended in the same buffer, containing 0 or 1 mM ATP, and incubated for 1 h at room temperature. Incubation mixtures were then centrifugated and residual nuclear pellets were analyzed by SDS–PAGE, together with nuclear extracts. As shown in Figure 8, incubation with buffer alone induced a partial release of the hsp 72 (but not of the hsp 66) from nuclei of heat-shocked cells. This release of the hsp 72 cannot be ascribed to a nonspecific leakage of nuclear material occurring during the incubation period, since the hsp 72 was the only protein detectable in the nuclear extract, with only a negligible background of other labeled proteins. When 1 mM ATP was added to the incubation buffer, the release of hsp 72 from nuclei was increased significantly. Again, the hsp 66 was not displaced from nuclei and was quantitatively recovered with the residual nuclear pellet. In four separate experiments, the total amount of hsp 72 released from nuclei either in the absence or in the presence of

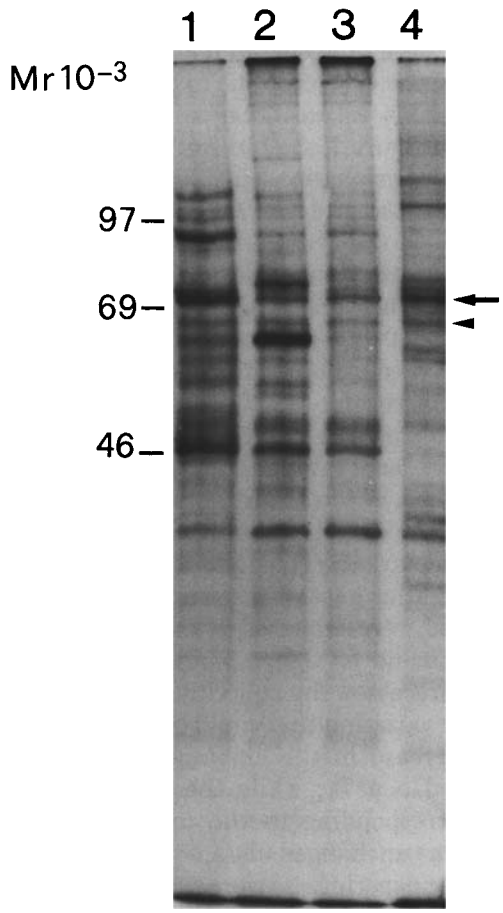

Fig. 7. Intracellular localization of hsp 66 in cells recovered from heat shock. Cultures were heated at 45°C for 20 min, returned to 37°C, and labeled with [^{35}S]methionine at 4–6 h post-treatment. After the pulse, the radioactive precursor was removed, and the recovery at 37°C was continued for additional 12 h (chase). At the termination of the pulse-chase period, cells were processed for subcellular fractionation as described in Materials and Methods. Proteins in the cytosol (*lane 1*), mitochondria (*lane 2*), microsome (*lane 3*), and nuclear fraction (*lane 4*) were analyzed by SDS–PAGE and fluorography. Note that, owing to the different radioactive content of the various subcellular fractions, the amount of material layered on the gel lanes was different on a per-cell basis: *lane 1*, loaded with 5×10^4 cell equivalents; *lanes 2,3,4,5*, loaded with 5×10^5 cell equivalents. The position of molecular-weight markers is reported on the left side. *Arrow (right)*, hsp 72; *arrowhead*, hsp 66.

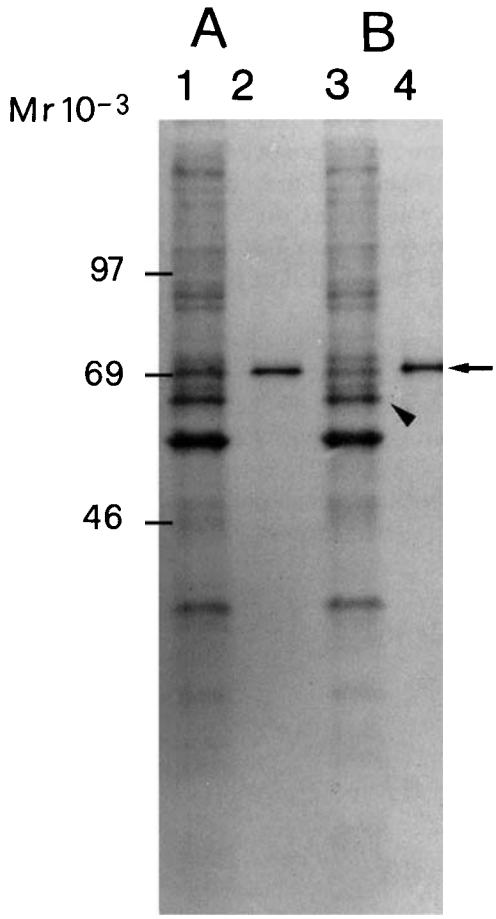

Fig. 8. Release of hsp 72 from nuclei incubated in vitro with or without ATP. Purified nuclear pellets were incubated for 1 h at room temperature with buffer alone (**A**) or with buffer supplemented with 1 mM ATP (**B**). Incubation mixtures were centrifuged and nuclear residual pellets (*lanes 1,3*), as well as soluble nuclear extracts (*lanes 2,4*) were analyzed by SDS–PAGE and fluorography. Aliquots of material corresponding to 1×10^6 nuclei were layered in all gel lanes. The position of molecular weight markers is reported on the left side. *Arrow (right)*, hsp 72; *arrowhead*, hsp 66.

ATP was remarkably constant: when nuclei were incubated with buffer alone, 12 (±2)% of the initial nuclear-associated radioactivity was released, while in the presence of ATP the amount of radioactivity recovered with the postnuclear supernatant increased to 16 (±2)%.

DISCUSSION

In some instances, it was observed that cells may respond in different ways to heat stress, depending on the severity of heat exposure. For example, in HeLa cells, it was observed that an unusual heat-shock protein of about 90 kDa was induced by treatment at 42°C, but not by treatment at 45°C: all the other major hsps, by contrast, were expressed at both these temperatures [Hatayama et al., 1986; Honda et al., 1988]. Conversely, in a number of mammalian cells, it was reported on a 40-kDa hsp synthesized at high rate only after extreme hyperthermic exposures [Margulis et al., 1991; Pipkin et al., 1992]. In keeping with these reports, in this paper we characterized a new hsp, the hsp 66, that is induced in M-14 human melanoma cells by

strong hyperthermic treatments (typically 45°C for 20 min), but not by mild hyperthermic treatments, sufficient to induce the other "classic" hsps. This new hsp 66, therefore, represents an additional example of hsp endowed with a thermal threshold for its induction markedly different than that of the other hsps.

A high-temperature-restricted expression of a 66-kDa protein was also found to occur in the human glioma cell line Li. By contrast, hsp 66 was not induced in seven other human cell lines (four originating from melanomas and three of different hystological origin). However, it should be considered that the effectiveness—in terms of cell survival—of the exposure at 45°C for 20 min greatly vary among the cell lines tested for hsp 66 induction. This represents a relevant point to explain the different behaviour of M-14 and Li cells with respect to the other cell lines, since the induction of the hsp 66 was not simply correlated with the temperature of the treatment, but rather with its effectiveness.

Immunological recognition experiments, performed making use of two recently developed MAbs—both able to recognize a single protein among the mammalian hsp 70 family—demonstrate that hsp 66 is antigenically related to hsp 72. A focal point is whether hsp 66 is genuinely novel or simply a definite breakdown product of hsp 72, originating from a specific degradation pathway activated in cells exposed to high temperatures. It could also be assumed that hsp 66 may arise from post-translational modifications of hsp 72. Both possibilities were ruled out by the results of the experiments reported in Figure 5, which showed that hsp 66 can be synthesized in rabbit reticulocyte lysates, as well as in *Xenopus* oocytes, programmed with Poly(A)$^+$ RNA obtained from cultures exposed at 45°C. In the same assays, Poly(A)$^+$ RNA extracted from mildly shocked cells was able to direct only the synthesis of hsp 72. These results clearly demonstrate the presence, in cells subjected to 45°C hyperthermia, of a mRNA coding for the hsp 66, distinct from the mRNA coding for the hsp 72. So far, four heat-inducible hsp 70 genes (hsps 70.1, 70.2, 70 B, and 70 B^1) have been recognized in mammalian cells [Lisowska et al., 1994]. All these genes are devoid of introns; therefore, hsp 66 cannot represent an alternatively spliced form of hsp 72. It is still to be clarified whether mRNA coding for hsp 66 originates from one of the above mentioned genes, to a minor start of translation site, or whether it is the product of a different, still unrecognized, gene.

A distinctive feature of the hsp 66 is that this protein does not bind to ATP-agarose. This behaviour was rather surprising, since proteins belonging to the hsp 70 family typically display strong ATP-affinity and this property has been exploited for their rapid purification on ATP-agarose [Welch and Feramisco, 1985]. Recent works demonstrate that there are two different conformations of hsp 72: an ADP-containing "high-affinity" form (tightly bound to the polypeptide substrate) and an ATP-containing "low-affinity" form (loosely bound to peptide substrate) [Craig et al., 1994]. It is likely that only the "low affinity" form is able to bind to ATP-agarose, by exchanging its own ATP with the immobilized nucleotide. In this context, it can be hypothesized that in cell lysates nearly all hsp 66 is present in a "high-affinity" conformation, and, therefore, it is unable to bind ATP in vitro.

Using cell fractionation methods, hsps 72 and 66 were found to be equally distributed among the different intracellular compartments of cells which recovered from heat shock: in particular, these proteins were found to be localized both in the cytoplasm and in the nucleus. A fraction of the nuclear-associated hsp 72 was released when purified nuclei were subjected to a prolonged incubation in buffer alone but a significantly greater amount of hsp 72 could be released from nuclei when ATP was present in the incubation buffer. In this regard, it should be noted that our samples were not subjected to any ATP-depleting treatment. Therefore, our nuclear preparations probably contained considerable amount of endogenous ATP, likely responsible for the partial release of hsp 72 occurring in nuclei incubated in ATP-free conditions. However, even in the presence of ATP, a substantial amount of hsp 72 was not extracted from nuclei and could be recovered with the residual nuclear pellet. A similar behaviour has been recently observed in heat-shocked HeLa cells, by using immunofluorescence staining [Ohtsuka et al., 1993]. With respect to the hsp 72, the hsp 66 appears to be much more tightly bound to nuclear structures, so that it was quantitatively retained inside nuclei, even following exhaustive incubation in the presence of ATP: this behaviour fits well with the inability of hsp 66 to be retained on ATP-agarose and with the fact that the pre-existing hsp 66 is not translocated

from cytoplasm to nuclei following an hyperthermic challenge, as hsp 72 does.

All the proteins belonging to the hsp 70 family typically display a chaperonine function, performed through binding to their protein target, followed by ATP-driven dissociation [Becker and Craig, 1994]. According to our results, hsp 66 does not seem to act in this way; on the contrary, this protein seems to bind steadily to cytoplasmic and nuclear structures. It is possible that in M-14 cells subjected to severe hyperthermic exposures hsp 66 specifically binds, in a tight and ATP-unreleasable way, to proteins too heavily damaged to be repaired, signalling and/or facilitating their disposal.

An attractive possibility is that the hsp 66 here described might correspond to the P 66 mot (mortalin) characterized by Wadhwa et al. [1993]. Beside having identical molecular weights, both these proteins are cross-reactive with the hsp 72 and are devoided of in vitro ATP-binding activity. Mortalin, however, was reported as not being heat-inducible, but the hyperthermic treatment applied to test heat inducibility (1 h at 42°C) was too mild for eliciting hsp 66 synthesis. In any case, until sequence analysis of the hsp 66 can be performed, this proposed identification of the hsp 66 with mortalin remains purely speculative.

ACKNOWLEDGMENTS

This work was supported by CNR grant 9400973CT04 (to A.D.) and by an A.I.R.C. fellowship (to P.P.).

REFERENCES

Becker J, Craig A (1994): Heat-shock proteins as molecular chaperones. Eur J Biochem 219:11–23.

Bigel F (1990): Use of *Xenopus* oocytes for functional expression of plasma membrane proteins. J Membr Biol 117:201–221.

Bonner WM, Laskey RA (1974): A film detection method for tritium-labelled proteins and nucleic acid in polyacrylamide gel. Eur J Biochem 46:83–88.

Craig EA, Weissman JS, Horwich AL (1994): Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. Cell 78:365–372.

Delpino A, Spinsanti P, Mattei E, Mileo AM, Vismara D, Ferrini U (1992): Identification of a 66 kD heat shock protein (HSP) induced in M-14 human melanoma cells by severe hyperthermic treatment. Melanoma Res 2:369-375.

Hatayama T, Honda K, Yukioka M (1986): HeLa cells synthesize a specific heat shock protein upon exposure to heat shock at 42°C but not at 45°C. Biochem Biophys Res Commun 137:957–963.

Honda K, Hatayama T, Yukioka M (1988): Characterization of a 42°C-specific heat shock protein of mammalian cells. J Biochem 103:81–85.

Konno A, Sato N, Yagihashi A, Torigoe T, Cho J, Torimoto K, Hara I, Wada Y, Okubo M, Takahashi N, Kikuchi K (1989): Heat or stress-inducible transformation-associated cell surface antigen on the activated H-ras oncogene-transfected rat fibroblast. Cancer Res 49:6578–6582.

Laemmli UK (1978): Cleavage of structural proteins during the assembly of the head of the bacteriophage T 4. Nature 274:699–701.

Lisowska K, Krawczyk Z, Widlak W, Wolniczek P, Wisniewski J (1994): Cloning, nucleotide sequence and expression of rat heat inducible hsp70 gene. Biochim Biophys Acta 1219:64–72.

Margulis BA, Zhivotovski BD, Pospelova TV, Smagina LV (1991): Patterns of protein synthesis in various cells after extreme heat shock. Exp Cell Res 193:219–222.

Ohtsuka K, Utsumi KR, Kaneda T, Hattori H (1993): Effect of ATP on the release of hsp 70 and hsp 40 from the nucleus in heat-shocked HeLa cells. Exp Cell Res 209:357–366.

Pipkin JL, Hinson WG, Lyn-Cook LE, Burns ER, Sheehan D, Casciano DA (1992): A nuclear protein associated with lethal heat shock of HL-60 cells. Exp Cell Res 202:167–173.

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Van Buskirk A, Crump BL, Margoliash E, Pierce SK (1989): A peptide binding protein having a role in antigen presentation is a member of the hsp 70 heat shock family. J Exp Med 170:1799–1809.

Wadhwa R, Kaul SC, Ikawa Y, Sugimoto Y (1993): Identification of a novel member of mouse hsp 70 family. Its association with cellular mortal phenotype. J Biol Chem 268:6515–6621.

Welch WJ (1990): The mammalian stress response: Cell physiology and biochemistry of stress proteins. In Morimoto R, Tissières A, Georgopoulos C (eds): "Stress Proteins in Biology and Medicine." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 223–278.

Welch WJ, Feramisco JR (1985): Rapid purification of mammalian 70,000-Dalton stress proteins: Affinity of the proteins for nucleotides. Mol Cell Biol 5:1229–1237.

Woodward RM, Polenzani L, Miledi R (1992): Effects of steroids on *o*-aminobutyric acid receptors expressed in *Xenopus* oocytes by poly(A)$^+$ RNA from mammalian brain and retina. Mol Pharmacol 41:89–103.