Identification of Two HSP70-related *Xenopus* Oocyte Proteins That Are Capable of Recycling Across the Nuclear Envelope

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Abstract. Two 70-kD polypeptides, B3 and B4, are present in equivalent concentrations in the nucleus and cytoplasm of *Xenopus* oocytes. The objectives of this study were to determine if they (*a*) are members of the 70-kD family of heat shock proteins, and (*b*) recycle between the nuclear and cytoplasmic compartments. Evidence based on high-affinity binding to ATP, cross-reactivity of B3/B4-specific antibodies with rat hsc70, and a comparison of cyanogen bromide cleavage peptide maps with hsc70, verified that B3 and B4 are members of the 70-kD family of heat-shock proteins. Nuclear uptake studies were performed by microinjecting ^{125}I-labeled B3/B4, rat hsc70, and BSA into the cytoplasm of oocytes, and examining their subsequent intracellular distributions. By 6 h postinjection, the nuclear concentration of B3/B4 and hsc70 were ~24-fold greater than BSA controls. It was also found that B3/B4-coated gold particles as large as 120Å in diameter were able to enter the nucleus by passing through the pores. Nuclear efflux was analyzed by microinjecting the iodinated proteins directly into the oocyte nuclei. 2 h after nuclear injection, at least 46% of the B3/B4 and 60% of the hsc70 were found in the cytoplasmic fractions, compared with <10% for the BSA controls. Cell fusion experiments, in which labeled, anucleate oocyte vegetal hemispheres were fused, under oil, with nucleate unlabeled animal hemispheres, demonstrated that cytoplasmic B3 and B4 could enter the nucleus after equilibration was reached, arguing against the existence of separate nuclear and cytoplasmic populations. Collectively, these results show that B3, B4, and rat hsc70 are transported across the nuclear envelope and recycle between the nucleus and cytoplasm.

Two prominent 70-kD *Xenopus laevis* oocyte proteins, designated B3 and B4 (pI 5.58 and 5.75, respectively), are present in equivalent concentrations in both the nucleus and cytoplasm (DeRobertis et al., 1978; Feldherr and Ogburn, 1980). These polypeptides are also present in adult *Xenopus* tissues, including liver, kidney, pancreas, brain, and heart (Dreyer and Hausen, 1983). Since B3 and B4 are not restricted to oocytes, it is likely that they have a universal role in cell function.

King and Davis (1987) and Horrell et al. (1987) suggested that B3 and B4 might be constitutively expressed heat shock proteins, similar to the heat shock cognates (hsc70s) characterized in *Drosophila* (Craig et al., 1983). These conclusions were based on similarities in the molecular weights and isoelectric points of these proteins.

Metabolically labeled B3 and B4 equilibrate between the nucleus and cytoplasm in ~3 h (Feldherr and Ogburn, 1980). Exogenous proteins of equivalent size that are known to enter the nucleus by passive diffusion require a much longer time to equilibrate (e.g., BSA; Bonner, 1975; Paine, 1975; Paine et al., 1975), indicating that the uptake of B3 and B4 is transport mediated. There are two likely explanations why these polypeptides do not accumulate in the nucleoplasm like other targeted oocyte nuclear proteins, such as nucleoplasmin (Dingwall et al., 1988) and N1/N2 (Kleinschmidt and Seiter, 1988). First, only a portion of the cytoplasmic pool of B3 and B4 might be available to the nucleus. This could be the result of either binding or compartmentalization within the cytoplasm. Second, B3 and B4 might shuttle between the nucleus and cytoplasm.

The objectives in this study were to determine whether B3 and B4 are constitutively expressed heat shock proteins, and if they are capable of recycling between the nucleus and cytoplasm. Both hsp70 and hsc70 are concentrated in the nucleus in response to heat or chemical stress (Welch and Feramisco, 1984; Pelham, 1984; Pelham, 1986) and apparently are redistributed back to the cytoplasm during recovery (Velaquez and Lindquist, 1984; Welch and Mizzen, 1988). It was not determined, however, whether these proteins are able to continuously recycle under nonstress conditions. Demonstrating that B3 and B4 are recycling heat shock cognate proteins could give useful clues as to the functions of this family of polypeptides, especially since they are so highly conserved (Lindquist and Craig, 1988). For example, recycling would be consistent with the hypothesis that these proteins act as carriers in signal-mediated nuclear transport. By analogy, hsp70 has been shown to be involved in the translocation of proteins across mitochondrial and endoplasmic reticulum membranes (Chirico et al., 1988; Deshaies et al., 1988).

We demonstrate, based on several lines of evidence, that B3 and B4 are 70-kD heat shock proteins. Microinjection and cell fusion studies provide evidence that B3 and B4 are transported across the nuclear envelope and are able to recycle between the nucleus and cytoplasm. Comparable experiments performed with rat hsc70 demonstrate that it behaves similarly to B3 and B4 when microinjected into oocytes. This suggests recycling could be a conserved property of the 70-kD heat shock cognates.

Materials and Methods

Xenopus laevis were purchased from Xenopus I, (Ann Arbor, MI), maintained in artificial pond water, and fed beef heart every other day. Ovaries were surgically removed from frogs anesthetized on ice. OR-2 medium (Wallace and Misulovin, 1978) was used as an extracellular medium in all experiments.

Isolation of B3, B4, and hsc70

B3 and B4 were isolated using a modification of the procedure described by Welch and Feramisco (1985). Approximately 25 ml of isolated ovary was homogenized in a Dounce homogenizer (Wheaton Instruments Div., Melville, NJ) on ice. An equal volume of an ice-cold solution containing 10 mM Tris-acetate pH 7.5, 10 mM NaCl, and 0.1 mM EDTA was added, and the homogenate was centrifuged at 100,000 *g* for 60 min at 4°C. The supernatant was clarified twice with 1,1,2-trichlorotrifluoroethane (Laskey et al., 1978). The remainder of the procedure followed that described by Welch and Feramisco except the ATP-agarose column was washed with 3 mM GTP before being eluted with 3 mM ATP. The ATP eluent was extensively dialyzed against 15 mM ammonium bicarbonate, and concentrated to 4 ml using an ultrafiltration cell (model 8010; Amicon Corp., Danvers, MA). It was then aliquoted into 0.5-ml fractions, frozen in dry ice/acetone, and lyophilized to dryness. One to 2 mg of protein was obtained from 25 ml of ovary.

Rat hsc70 was purified from brains collected from three freshly guillotined rats. The procedure of Welch and Feramisco was followed except the DEAE step was omitted.

Purity of both B3 and B4 and rat hsc70 was confirmed by two-dimensional isoelectric focusing PAGE (O'Farrell and O'Farrell, 1977). The proteins were stored dry at −20°C.

Since B3 and B4 copurify, the isolate will be referred to in the subsequent discussion as B3/B4.

Antibodies

To generate polyclonal antibodies, two female New Zealand White rabbits were immunized subcutaneously every 14 d with B3 or B4 protein spots cut from unfixed two-dimensional gels of whole oocyte protein. Before injection, the gel spots were homogenized in RIBI adjuvant system (Immunochem Research Inc., Hamilton, MT). Approximately 20 μg of B3 or B4 was used for each immunization. After the third immunization, Western blots of two-dimensional gels of oocyte protein (Towbin et al., 1979) were probed with serum from each rabbit. Both polyclonal antisera reacted with B3 and B4. Polyclonal antibodies were also prepared against isolated B3/B4 using the same injection regimen described above. Approximately 20 μg of isolated B3/B4 were used for each immunization. The antibodies were purified by passing sera over an affinity column made by conjugating purified B3 and B4 with CNBr-Sepharose 4B (Sigma Chemical Co., St. Louis, MO). The column was washed with 20 mM Tris-HCl (pH 7.2), 300 mM NaCl, and bound antibodies were eluted by a low pH wash (0.1 M glycine-HCl, pH 2.6, 500 mM NaCl). The eluent was dialyzed into 20 mM Tris-HCl pH 7.2, 100 mM NaCl, and the IgGs were purified using Protein A-Sepharose (Sigma Chemical Co.).

Cyanogen Bromide–Cleavage Peptide Mapping

Cyanogen bromide-cleavage peptide mapping was performed using a modification of the procedure described by Sokolov et al. (1989). Approximately 50 μg of purified B3 and B4, and 25 μg of purified rat brain hsc70 were resolved on two-dimensional gels. The proteins were visualized by staining unfixed gels for 15 min with 0.0625% Coomassie blue in 25 mM KCl, 3 mM NaCl, 1.8 mM K_2HPO_4, 1.2 mM KH_2PO_4, pH 7.0, and destaining in deionized water for 5 min. B3, B4, and hsc70 spots were cut from the gels, fragmented and lyophilized to dryness. 45 μl (per gel spot) of cyanogen bromide (CNBr;[1] 200 mg/ml in 70% formic acid) was added to tubes containing B3, B4, or hsc70. The gel fragments were incubated for 12 h at 37°C, then lyophilized to dryness. They were then rehydrated with 100 μl deionized water, and relyophilized. This step was repeated twice. 40 μl (per spot) of complete sample buffer at pH 7.2 (Laemmli, 1970) containing bromophenol blue was added to each tube. After a 10-min incubation period (at 21°C), the samples were buffered with microliter aliquots of 3 M NaOH until the indicator turned from yellow/green to blue. The fragments were then run at the equivalent of 2 protein spots per lane on a 12% SDS-polyacrylamide gel (Laemmli, 1970). They were then electrophoretically transferred to a polyvinylidine difluoride (PVDF) membrane (Immobilon; Millipore Corp. Milford, CA) in 20 mM 3-[cyclohexamino]-1-propanesulfonic acid (Sigma Chemical Co.; pH 9.9, dissolved in 10% methanol). The blots were stained with 0.1% Coomassie blue (Serva Fine Biochemicals, Garden City Park, NY) in 80% methanol and air dried.

Protein Sequencing

CNBr-cleaved peptide fragments were fractionated and resolved as described above. Fragments to be sequenced were cut from the dried Immobilon membrane and applied directly to a gas-phase amino acid sequencer with an online phenylthiohydantoin analyzer (model 470A; Applied Biosystems Inc., Foster City, CA.). Sequencing was performed by the Protein Chemistry Core Facility at the University of Florida.

Protein Iodination

Approximately 40 μg of purified B3/B4, rat hsc70, or BSA (Sigma Chemical Co.) was reacted with Bolton and Hunter reagent (Amersham Chemical Co., Arlington Heights, IL; Bolton and Hunter, 1973). The labeled proteins were separated from uncoupled label using a Sephadex G-25 column equilibrated with intracellular medium, which contains 102 mM KCl, 11.1 mM NaCl, 7.2 mM K_2HPO_4, 4.8 mM KH_2PO_4 (Feldherr and Pomerantz, 1978). The average specific activities for B3/B4, hsc70, and BSA were ~5.0 × 10^7, 6.3 × 10^7, and 1.0 × 10^9 cpm/nmol, respectively. For B3/B4, hsc70, and BSA, respectively, ~4,500, 4,000, and 80,000 cpm were injected into the cytoplasm, and 450, 400, and 5,400 cpm were injected into the nucleus.

Microinjections

Microinjection experiments were performed using manually defolliculated late stage 5 or stage 6 oocytes (Dumont, 1972). For cytoplasmic injections, the pipettes were calibrated to deliver ~50 nl of solution. The injectates, ~4 ng of labeled protein in intracellular medium, were directed into the vegetal hemisphere of the oocytes to avoid accidental contact with the nucleus. It is estimated that the amount of protein injected represented between 0.8 and 2.0% of the total cellular B3/B4. For nuclear injections, cells were centrifuged for 8–10 min at ~650 *g* (Kressman and Birnsteil, 1980; Feldherr et al., 1984). As a result of centrifugation, the nucleus migrates to the inner surface of the animal pole and, due to the displacement of pigment granules, can be readily identified. Nuclei were injected with 5 nl of the protein solutions. All microinjection experiments were carried out at 21°C in OR-2 medium.

Quantitation of Nuclear Uptake of Injected Proteins

To measure the nuclear uptake rates of B3/B4 and rat hsc70, iodinated proteins were microinjected into cytoplasm of oocytes, and the cells were manually enucleated 1, 2, 3, 6, and 15 h postinjection (20 cells per time point). ^{125}I-BSA controls were injected and enucleated after 6 and 15 h. Isolated nuclei and cytoplasms were transferred into 95% ethanol immediately after fractionation (Feldherr and Richmond, 1977). To determine the amount of label in nuclear and cytoplasmic fractions at each time point, ^{125}I was measured in pooled fractions using a Packard Auto-Gamma Scintillation Spectrometer (model 5110; Packard Instruments Co., Downers Grove, IL). To determine if there was breakdown of the injected proteins and also confirm the quantitation obtained by gamma counting, aliquots of nuclear and cytoplasmic fractions were analyzed by one-dimensional gel autoradiography

1. *Abbreviations used in this paper*: CNBr, cyanogen bromide; PVDF, polyvinylidine difluoride.

(Towbin et al., 1979), and quantitated by densitometry (soft laser scanning densitometer, model SLR-2D/1D; Biomed Instruments, Fullerton, CA). Each lane was analyzed separately to determine the amount of breakdown in the individual cellular compartments. The linear range for densitometric analysis was established using standard curves of three to four different time exposures of the same gel.

Quantitation of Nuclear Efflux of Injected Proteins

To study nuclear efflux, we injected iodinated B3/B4, rat hsc70 or BSA directly into oocyte nuclei. 2 h postinjection, cells were enucleated and both nuclear and cytoplasmic fractions were immediately transferred to 95% ethanol. ^{125}I was measured in individual nuclei and their corresponding cytoplasms as described above. Analysis of individual cells made it possible to distinguish between successful injections, in which the injectate was introduced entirely into the nucleus, and unsuccessful injections in which there was leakage into the cytoplasm. Efflux experiments were performed for 2 h to minimize the long-term effects of puncturing the nuclear envelope.

Aliquots of nuclei and cytoplasms were also analyzed by one-dimensional gel autoradiography, as described above, to determine if there was breakdown of the injected protein and to confirm the results obtained by direct gamma analysis.

Measurement of Endogenous Nuclear and Cytoplasmic Concentrations of B3 and B4

To determine the intracellular distribution of B3 and B4, double-label analysis was used as described previously (Feldherr and Ogburn, 1980) with some modifications. Experimental cells were metabolically labeled with [^{3}H]leucine and cell fractions were simultaneously electrophoresed with nuclei isolated from [^{35}S]methionine-labeled cells. The ^{35}S-labeled nuclei served as internal standards. To avoid labeling the surrounding follicle cells that are known to contain hsp70 (Horell et al., 1987), 40 nl of intracellular medium containing 1 μCi of [^{3}H]leucine (143 Ci/mMol) was injected into the cytoplasm of oocytes, and the cells were immediately transferred to OR-2 medium containing 3 mM cold leucine to dilute any labeled amino acids that may have leaked from the oocytes after injection. Cells were transferred to OR-2 medium without leucine after 2 h. The oocytes were enucleated 9 h postinjection, and the nuclei and cytoplasms were fixed in 95% ethanol. Both fractions were resolved on two-dimensional gels, and B3 and B4 protein spots were excised. Radioactivity was measured using a liquid scintillation counter (L55000D; Beckman Instruments), and the $^3H/^{35}S$ ratios were determined.

Stabilization of Gold Colloid

Colloidal gold particles 20–120 Å in diameter were prepared by reducing chloroauric acid with a saturated solution of white phosphorous in ether (Feldherr, 1965). The gold sols were stabilized with B3/B4, concentrated, dialyzed, and microinjected as described by Dworetzky and Feldherr (1988). Fixation and electron microscopic analysis were also performed according to Dworetzky and Feldherr (1988).

Effect of Temperature on Uptake of B3 and B4

Oocytes were injected cytoplasmicly with ^{125}I-B3/B4 or ^{125}I-myoglobin and were either incubated at 21°C or immediately transferred to OR-2 at 4°C. 14–17 oocytes were injected in each experimental group. 3 h after injection the oocytes were enucleated, and the radioactivity in isolated nuclear and cytoplasmic fractions was determined as described above.

Hemicell Fusion Experiments

^{125}I-labeled B3/B4 or ^{125}I-labeled BSA was microinjected into the cytoplasm of defolliculated oocytes. The cells were allowed to equilibrate for 18 h at room temperature in OR-2 medium. Following the procedure of Feldherr et al. (1988), injected cells were then bisected under oil along their animal-vegetal boundaries. Labeled vegetal hemispheres were then fused with unlabeled (nucleated) animal hemispheres. After 4 h, nuclei were isolated and both nuclear and cytoplasmic fractions were fixed in 95% ethanol and analyzed for ^{125}I. One-dimensional gel autoradiography was also performed to control for breakdown of the labeled protein and also confirm the quantitation obtained from the gamma counter. The values for B3/B4 and BSA were obtained from seven and eight fusions, respectively.

Results

Isolation and Characterization of B3, B4, and Rat hsc70

A typical Coomassie blue–stained two-dimensional gel obtained for *Xenopus* oocyte nuclei is shown in Fig. 1 *A*. B3 and B4 have apparent molecular masses of 70 kD, and pIs of 5.58 and 5.75, respectively.

A Coomassie blue–stained two-dimensional gel of proteins purified from whole oocyte 100,000 *g* supernatant using ATP affinity chromatography is shown in Fig. 1 *B*; two major polypeptides are apparent. Based on their apparent molecular masses (70 kD), pIs (5.6 and 5.8), and reactivity with B3- and B4-specific polyclonal antibodies (Fig. 1 *C*), we concluded that these isolated polypeptides are B3 and B4.

A two-dimensional gel of the purified rat hsc70 is shown in Fig. 2. One major species is present, with an apparent molecular mass of 70 kD and a pI of 5.65. These values are consistent with those reported for hsc70 (Pelham, 1984, 1986; Lindquist and Craig, 1988). Its identity was confirmed by comparing the amino-terminal sequence of a 31-kD CNBr-cleavage peptide fragment (see below) to the predicted sequence of hsc70 derived from a full-length cDNA clone (O'Malley et al., 1985). The two sequences are compared in Table I. Affinity-purified anti B3/B4-specific polyclonal antibodies were found to be strongly cross-reactive with isolated hsc70 (Fig. 2, *inset*). The minor spots present in the gel shown in Fig. 2 are probably impurities since they do not react with anti-B3/B4 antibodies. In addition, these antibodies were found to be specific for hsc70 when two-dimensional Western blots of rat brain 100,000g supernatant were probed (data not shown).

CNBr-cleavage mapping studies were performed with isolated B3, B4, and hsc70. The results are shown in Fig. 3. The patterns obtained for the three polypeptides are indistinguishable.

Nucleocytoplasmic Distribution of Endogenous B3 and B4

Double-labeling experiments were performed to establish the intracellular distribution of endogenous B3 and B4. The results of two experiments, performed on groups of 15 and 18 cells, are shown in Table II. We found that an average of 9.5% of the endogenous B3 and B4 are localized in the nucleus at equilibrium. Correcting for yolk content, the nucleus occupies approximately 12% of the total cell volume (Bonner, 1975).

Nuclear Uptake of ^{125}I-labeled BSA, B3, and B4

Fig. 4 *A* shows the data from three separate ^{125}I-B3/B4 microinjection experiments. Equilibration of labeled B3 and B4 was nearly complete by 6 h postinjection, and at 15 h ~9.0% of the total injected ^{125}I-labeled B3/B4 was present in the nucleus. The uptake kinetics are consistent with those reported for endogenous B3 and B4 (Feldherr and Ogburn, 1980). The intracellular distribution is also similar to that obtained for the endogenous species (Table II). Thus, the labeled tracers reflect the migration patterns of endogenous B3/B4. Nuclear uptake of ^{125}I-labeled B3/B4 at equilibrium

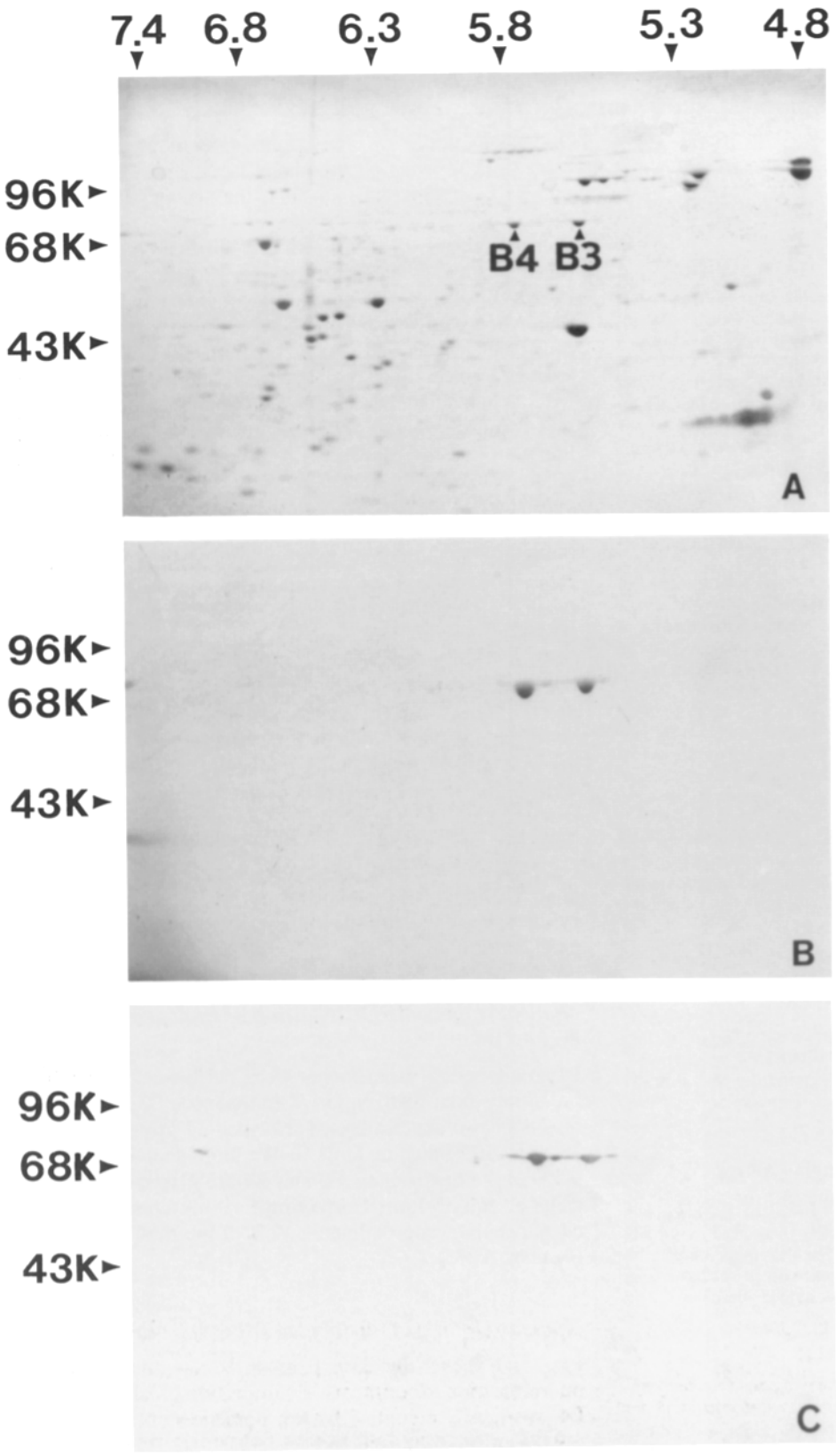

Figure 1. Purification of B3 and B4. *A* shows a two-dimensional gel of proteins from 40 oocyte nuclei stained with Coomassie blue. The positions of B3 and B4 are indicated. Isoelectric points and molecular weights are indicated along the abscissa and ordinates, respectively. *B* shows a Coomassie blue–stained two-dimensional gel of B3 and B4 purified from whole-oocyte 100,000-*g* supernatant by ATP affinity chromatography. *C* shows a Western blot of a two-dimensional gel of 40 oocyte nuclei probed with anti-B3/B4 rabbit serum (1:100 dilution). Antibodies were visualized using alkaline phosphatase–conjugated goat-anti–rabbit secondary antibodies (Sigma Chemical Co.). The small labeled spots adjacent to the major spots are most likely isoforms of B3 and B4. Identical results were obtained using either anti-B3 or anti-B4.

was ~25-fold greater than that observed for microinjected ^{125}I-BSA (9.1 vs. 0.36%; see Fig. 4 *A*).

Fig. 5 *A* is an autoradiograph of a one-dimensional gel of nuclear and cytoplasmic aliquots from injected cells. There was no significant breakdown of the labeled proteins. Densitometric analysis demonstrated that >95% of the labeled proteins in the cells had an apparent molecular mass of 70 kD. A minor 68-kD species is present in the cytoplasmic, but

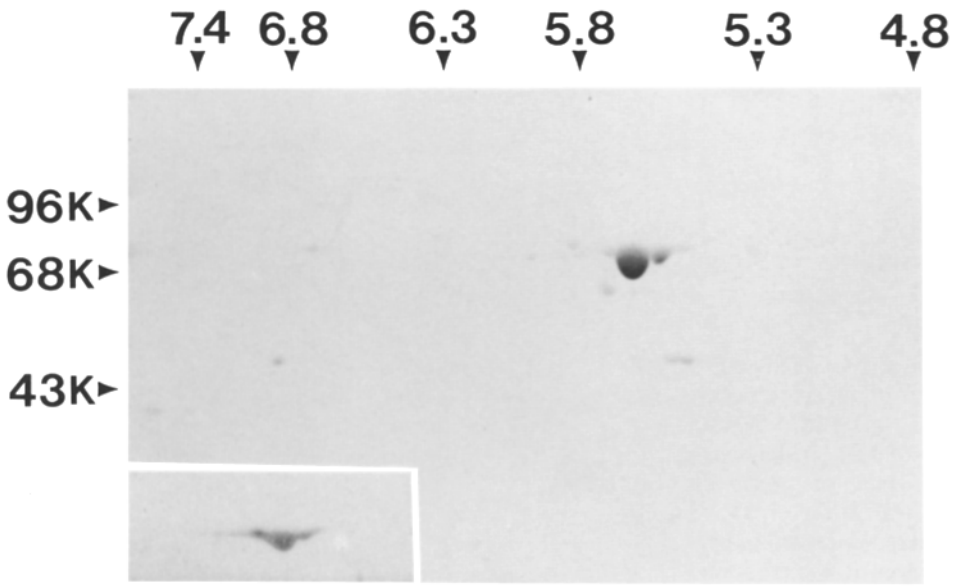

Figure 2. Purification of rat hsc70. Shown is a Coomassie blue-stained two-dimensional gel of purified rat hsc70. Isoelectric points and molecular weights are indicated along the abscissa and ordinate, respectively. (*Inset*) A Western blot of a two-dimensional gel of purified rat hsc70 probed with anti-B3/B4 rabbit serum (1:100 dilution). Antibodies were visualized as described for Fig. 1 *C*.

not nuclear aliquots. Whether this represents a breakdown product of B3 and B4 (Mitchell et al., 1985) or a minor contaminant is unclear. The exclusion of this 68-kD polypeptide from the nucleus while B3 and B4 are rapidly taken up is consistent with the view that translocation of these proteins is a transport-mediated process. The inset in Fig. 5 *A* is an autoradiograph of a two-dimensional gel that shows that equivalent amounts of microinjected ^{125}I-labeled B3 and B4 enter the nucleus. This is consistent with the data obtained for endogenous B3 and B4 (see Table II).

Nuclear Uptake of ^{125}I-labeled hsc70

Uptake experiments identical to those described for B3/B4 were performed using hsc70 (Fig. 4 *B*). The nuclear uptake rates of hsc70 were similar to those obtained for B3/B4, and were ~24-fold greater than observed for BSA. Fig. 5 *B* shows no significant breakdown of the injected hsc70; >95% of the labeled protein remained intact throughout the course of the experiment as determined by densitometric analysis.

Nuclear Efflux of ^{125}I-labeled B3 and B4

Nuclear efflux was measured by injecting ^{125}I-labeled protein directly into oocyte nuclei and measuring the amount that subsequently entered the cytoplasm. Fig. 6, *A* and *B* are distribution graphs, showing the percent of nuclear-injected BSA and B3/B4 found in the cytoplasm of individual cells 2 h postinjection.

^{125}I-BSA controls (Fig. 6 *A*) show that in approximately 70% of the cells, <10% of the injected BSA entered the cytoplasm. Since nuclear uptake and efflux of BSA occurs by passive diffusion (Bonner, 1975; Paine, 1975; Paine et al., 1975; Lanford et al., 1986), it is expected that only a few percent of the injected BSA would passively diffuse from the nucleus in 2 h. Therefore, we considered the 0–10% distribution peak to represent successful nuclear injections, and values greater than 10% representative of partial or missed injections.

Results of nuclear injections of B3/B4 (Fig. 6 *B*) show that in ~70% of the cells, 30–70% of the labeled protein had entered the cytoplasm after 2 h. Values >70% were considered partial or missed nuclear injections. The means and standard deviations for the amount of injected BSA and B3/B4 found in the cytoplasm of the groups containing 70% of the cells were 6.2 ± 1.1 and 46 ± 5.7%, respectively. If the 50–60% values for B3/B4 efflux are excluded from the calculations (i.e., considered to be unsuccessful injections), the lowest efflux rates obtained for B3/B4 are still significantly greater than the values obtained for BSA, indicating that B3/B4 leave the nucleus faster than predicted by passive diffusion.

Fig. 7, *A* and *B* shows autoradiographs of one-dimensional gels from two separate nuclear injection experiments. Fig. 7 *A* shows an aliquot of B3/B4 injected nuclei from cells found within the 30–60% distribution peak (lane *1*), and an aliquot from ten pooled cytoplasms from the same cells (lane *2*). Fig. 7 *B* shows aliquots from fractions identical to those

Table I. NH_2-Terminal Sequence Analysis of the 31-kD CNBr Cleavage Fragment of Rat hsc70

Obtained from 31-kD fragment	V N * F I A * F K * K * K K
Predicted from cDNA	V N H F I A E F K R K H K K

* The amount of material was not sufficient to resolve these residues.

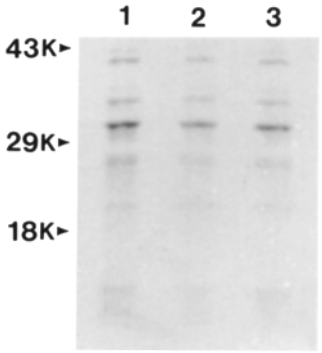

Figure 3. Cyanogen bromide cleavage maps of rat hsc70 and oocyte B3 and B4. Digested peptides were resolved on a 12% SDS-polyacrylamide gel, then electrophoretically transferred to a PVDF membrane and stained with Coomassie blue. The patterns obtained for rat hsc70 (lane *1*), oocyte B3 (lane *2*), and B4 (lane *3*) are not distinguishably different.

Table II. Intracellular Distribution of Endogenous B3 and B4

Protein	Exp. 1	Exp. 2
	*% of total protein in the nucleus**	
B3	6.4	10.7
B4	10.1	11.0

* These results were obtained 9 h after radiolabeling. Based on the data shown in Fig. 4 *A*, equilibrium between the nucleus and cytoplasm should have been achieved by this time.

in *A* (lanes *1* and *2*), and, in addition, an aliquot of 10 pooled cytoplasms from cells in which 70–90% of the label was cytoplasmic (lane *3*). In addition to the 70-kD B3/B4 band, a lower molecular weight polypeptide (~68 kD), analogous to that seen in the nuclear uptake experiments, was present in the injected nuclei (this is especially clear in Fig. 7 *A*) and cytoplasmic aliquots from cells considered to represent partial or missed nuclear injections. However, it was not found in cytoplasmic fractions from successful nuclear injections, supporting our earlier conclusion that it does not cross the nuclear envelope. Since this protein is nonexchangeable, it can serve as a marker for the site of injection, and its distribution is consistent with our interpretation of successful vs. partial or missed nuclear injections. The proportion of the 68-kD species, and its migration pattern, was found to vary in different experiments, probably the result of using different preparations of B3/B4.

Nuclear Efflux of hsc70

Nuclear efflux of hsc70 was measured as described above for B3 and B4. Results of nuclear injections of hsc70 (Fig. 6 *C*) show that in ~70% of the cells, 50–70% of the labeled protein had entered the cytoplasm after 2 h. Values >70% were considered bad injections. The mean and standard deviation for the cytoplasmic concentration of hsc70 in the successful experiments was 60 ± 4.4%. The lowest efflux rates obtained for hsc70 (50–60%) are still significantly greater than the values obtained for BSA, demonstrating that hsc70 also leaves the nucleus faster than expected for passive diffusion.

The means of the efflux values from successful hsc70 and B3/B4 nuclear injections are significantly different. Although the precise interpretation of this result is not clear, it could reflect differences in signal efficiency, or the interaction of B3/B4 with oocyte nuclear components.

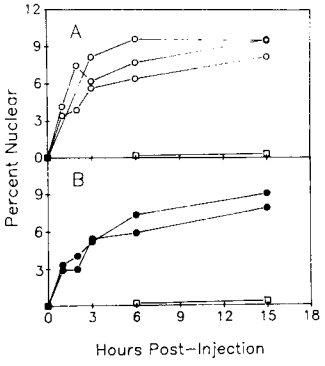

Figure 4. Nuclear uptake kinetics of ^{125}I-labeled B3/B4 (*A*) and ^{125}I-labeled rat hsc70 (*B*). *Open circles*, B3/B4; *closed circles*, hsc70; *open squares*, BSA controls. Each curve represents an independent experiment.

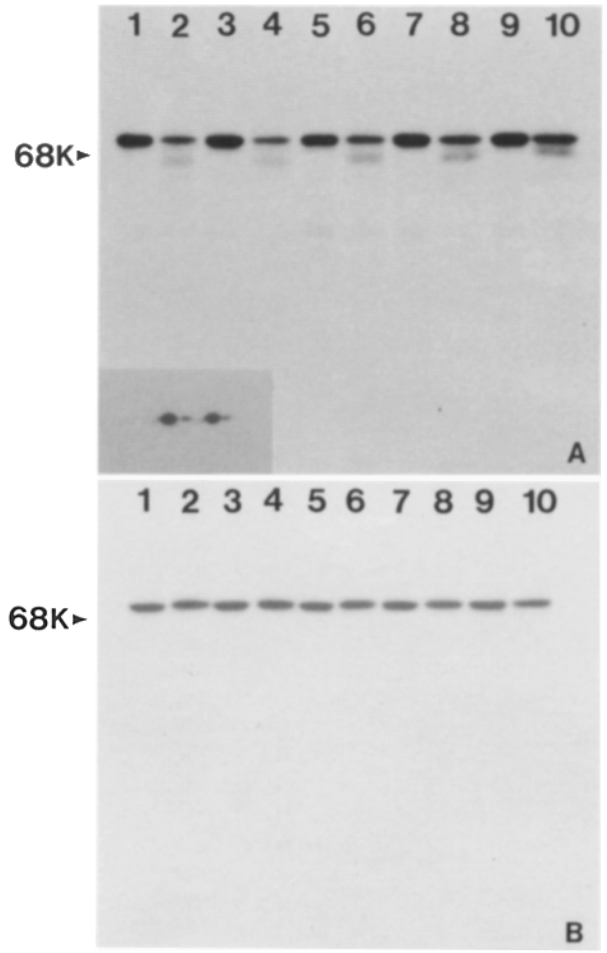

Figure 5. Autoradiographs of one-dimensional gels of nuclear and cytoplasmic fractions after cytoplasmic injection of ^{125}I-B3/B4 (*A*) and ^{125}I-hsc70 (*B*). In both *A* and *B*, equivalent counts were applied to each lane of a one-dimensional gel from fractions taken 1 (lanes *1* and *2*), 2 (lanes *3* and *4*), 3 (lanes *5* and *6*), 6 (lanes *7* and *8*), and 15 (lanes *9* and *10*) h after injection. Nuclear fractions are shown in lanes *1*, *3*, *5*, *7*, and *9*, and cytoplasmic fractions are shown in lanes *2*, *4*, *6*, *8*, and *10*. It has not been established whether the minor lower molecular weight bands seen in *A* are breakdown products or impurities in the preparation. (*Inset*) Autoradiograph of a two-dimensional gel showing labeled B3 and B4 from a nuclear fraction 6 h after a cytoplasmic injection. The migration pattern is indistinguishable from that observed for the isolated protein shown in Fig. 1 *B*.

Colloidal Gold Uptake and Temperature Effects

The intracellular distribution of B3/B4-coated gold particles 30 min after cytoplasmic injection is illustrated in Fig. 8. Gold is seen associated with the cytoplasmic surface of the nuclear pores and extending through the centers of the pores. Particles >120 Å in diameter (including the coat material) can readily penetrate the nuclear envelope. The size distribution of the particles located in the nucleus and cytoplasm from three cells are shown in Table III. Chi-square analysis confirmed that the two populations are not significantly different ($P = 0.9$). The N/C particle ratio is 0.18, ~30- and 20-fold greater than that obtained for particles of similar size coated with polyvinylpyrrolidone (PVP) (Feldherr et al., 1984), and BSA (Dworetzky et al., 1988), respectively.

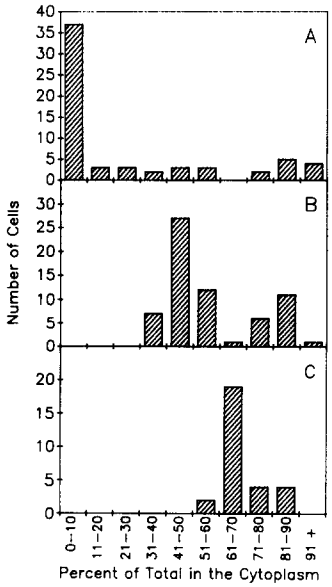

Figure 6. Nuclear efflux kinetics of ^{125}I-labeled BSA (*A*), B3/B4 (*B*), and hsc70 (*C*). Single-cell analysis was performed with the number of cells indicated on the ordinates and percent of injected protein located in the cytoplasmic fraction 2 h after nuclear injection indicated on the abscissa.

Signal-mediated transport into the nucleus has been shown to be a temperature-dependent process (e.g., Newmeyer et al., 1986*a*; Richardson et al., 1988). To determine the effect of temperature on the nuclear uptake of B3/B4, the intracellular distribution of microinjected ^{125}I-labeled B3/B4 was compared in cells maintained at 21 and 4°C. The percent incorporation of labeled B3/B4 into the nucleus after 3 h was 6.5 and 2.8%, respectively, which represents a 2.3-fold reduction. For comparison, the nuclear incorporation of a diffusible molecule, ^{125}I-labeled myoglobin (17.8 kD) (see Bonner, 1975; Paine et al., 1975), was also studied. 12.3 and 15% of the injected myoglobin was present in the nucleoplasm after 3 h at 21 and 4°C, respectively. Although the effect of temperature on B3/B4 transport is not as great as observed for other nuclear proteins (i.e., nucleoplasmin, see Newmeyer et al., 1986*a*), the results are significantly different than those obtained for passive diffusion.

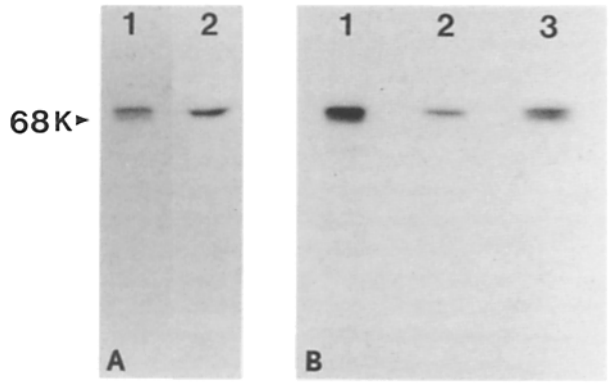

Figure 7. Autoradiographs of one-dimensional gels of nuclear and cytoplasmic fractions 2 h after nuclear injections of ^{125}I-B3/B4 from two separate experiments (*A* and *B*). Aliquots were applied to each lane of a 10% SDS-polyacrylamide gel. (*A*) Lane *1* is a nuclear aliquot from cells in which 30 to 60% of the label was cytoplasmic (successful injections). Lane *2* is a cytoplasmic aliquot from the same cells. (*B*) Lanes *1* and *2* are the same as those in *A*, but from a separate experiment. Lane *3* is a cytoplasmic aliquot from cells in which 80% of the injected label was cytoplasmic.

Hemicell Fusion Experiments

The nuclear uptake of ^{125}I-B3/B4 after fusion of labeled vegetal hemispheres from equilibrated cells with unlabeled animal hemispheres was significantly greater than that observed for ^{125}I-BSA controls (5 vs. 1%; $P < 0.001$ by t test). The higher than expected value for the nuclear uptake of BSA 0.19% (expected) vs. 1.0% (observed), reflects error inherent in the experimental procedure, possibly the result of cytoplasmic contamination of the isolated nuclei, or damage to the nuclear envelope during the nuclear isolation procedure. These factors are more difficult to control during oil isolation compared with aqueous isolation procedures.

Discussion

In this study, we obtained data that show the following: (*a*) B3 and B4 have molecular weights and isoelectric points consistent with those established for hsp70-related polypeptides (Pelham, 1986; Lindquist and Craig, 1988); (*b*) B3 and B4 bind with high affinity to ATP, a property shared by hsp70-related proteins (Welch and Feramisco, 1985; Chappell et al., 1986; Lindquist and Craig, 1988); (*c*) B3- and B4-specific polyclonal antibodies cross-react with a well-characterized, constitutively expressed rat heat shock protein, hsc70; (*d*) a comparison of CNBr-cleavage maps of B3, B4, and hsc70 reveals no distinguishable differences; (*e*) B3, B4, and hsc70 have similar nucleocytoplasmic distributions when microinjected into the oocyte. These results provide conclusive evidence that B3 and B4 are members of the 70-kD family of heat shock proteins.

It was also found, based on microinjection studies using isolated, radiolabeled protein, that B3 and B4 are capable of bidirectional exchange across the nuclear envelope. Several independent lines of evidence suggest that the translocation of B3/B4 is signal mediated. First, they enter and leave the nucleus faster than BSA, which lacks a nuclear localization signal and, thus, serves as a standard for the passive diffusion of polypeptides in the 70-kD range. Second, B3/B4 can initiate the uptake of colloidal gold particles over 120 Å in diameter through the nuclear pores. These particles are appreciably larger than the 90-Å exclusion limit observed for nontargeted molecules (Paine et al., 1975). And third, the uptake of B3/B4 is temperature dependent. Collectively, these results support the conclusion that the translocation of B3/B4 across the nuclear envelope is transport mediated. Similar intracellular distribution patterns were obtained for rat hsc70, indicating that it is also transported between the nucleus and cytoplasm.

The cell fusion experiments demonstrate that cytoplasmic B3 and B4 enter the nucleus even after equilibration of the labeled population, which argues against the existence of separate nuclear and cytoplasmic populations. These results, and the evidence that transport is bidirectional, strongly support the conclusion that B3 and B4 are capable of continuously recycling between the nucleus and cytoplasm.

Domains necessary for the nuclear and nucleolar localization of human hsp70 have been identified by Milarski and Morimoto (1989). A specific amino acid sequence, K-R-K-

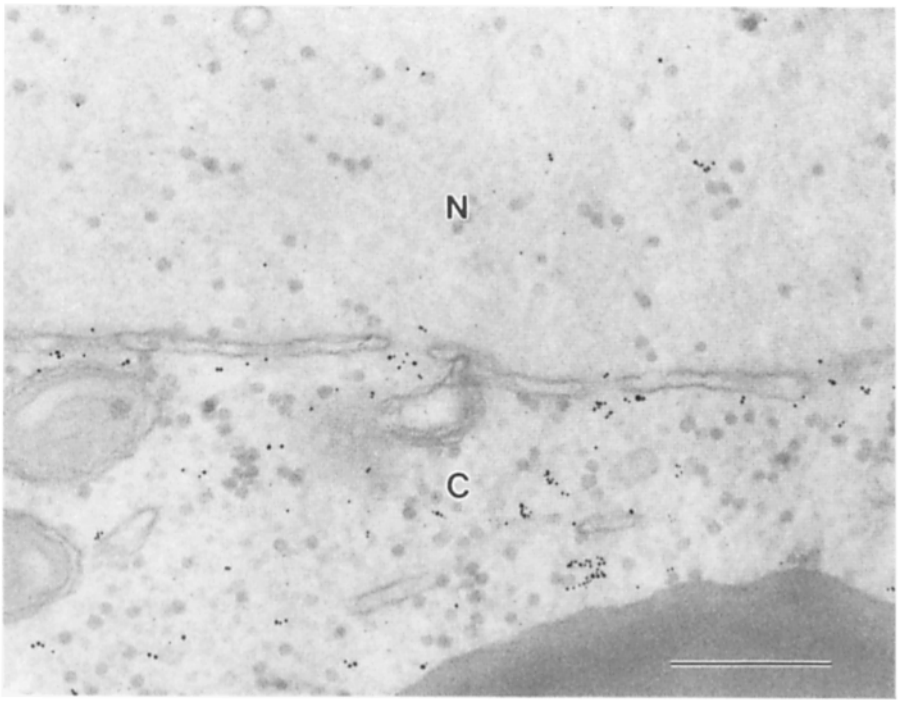

Figure 8. The intracellular distribution of B3/B4-coated gold, 30 min after a cytoplasmic injection. Particles are present in the cytoplasm (*C*) and the nucleus (*N*), and also adjacent to and within the nuclear pores. Bar, 0.5 μm.

H-K-K-D-I-S-Q-N-K-R-A-V-R-R, is sufficient to target pyruvate kinase fusion proteins to the nucleus and nucleoli of COS7 cells (Dang and Lee, 1989). This sequence is conserved in rat hsc70 except for a single amino acid substitution of glutamine for glutamic acid (O'Malley et al., 1985). Since this substitution does not involve a highly basic amino acid (e.g., arginine or lysine), it would not be expected to alter the effectiveness of the sequence as a nuclear targeting signal (see Lanford et al., 1988; Underwood and Fried, 1990). Although the genes for *Xenopus* B3 and B4 are not yet cloned, the highly conserved nature of the hsp70-related proteins suggests that a similar localization signal might be present in B3 and B4.

The nuclear uptake of large karyophilic proteins that are unable to passively diffuse across the nuclear envelope is an energy-dependent, signal-mediated process (for review, see Dingwall and Laskey, 1986). However, little is known about the efflux of proteins from the cell nucleus. Paine (1975) compared the movement of different sized exogenous tracers across the nuclear envelope of salivary gland cell nuclei and found similar diffusion rates both into and out of the nucleus. Lanford et al. (1986) further demonstrated that proteins effectively too large to passively diffuse into the nucleus, such as BSA and IgG, do not diffuse out. These findings suggest that, analogous to protein import, the nuclear efflux of large proteins (e.g., B3, B4 and hsc70) is a signal-mediated process. To date, no targeting sequences have been described for the nuclear efflux of any proteins. Nuclear uptake signals do not appear sufficient to initiate the transport of polypeptides from the nucleus to the cytoplasm (Feldherr et al., 1984; suggested by Newmeyer et al., 1986*a*,*b*), which suggests that efflux signals are separate and distinct from uptake signals. Since B3, B4, and rat hsc70 are apparently recycling proteins, they are good models for the study of nuclear efflux. We have recently obtained a full length cDNA clone of rat hsc70 (Green and Liem, 1989) and are attempting to perform deletion analyses to define peptide domains necessary for nuclear efflux.

Table III. Size Distribution of Gold Particles in the Cytoplasm and Nucleus of Cells 30 min Post Injection

	Total no. of particles measured	Percentage of particles in each size (diameter) class*				
		20–40 Å	40–60 Å	60–80 Å	80–100 Å	100–120 Å
Nucleus	792	11.4	40.0	33.8	11.9	2.0
Cytoplasm	797	10.8	42.0	34.5	9.3	2.7

* Particle dimensions do not include the thickness of the protein coat. The coat material adds ~30 Å to the overall diameter (Dworetzky and Feldherr, 1988).

Recycling would be an expected property of carriers involved in the transport of other macromolecules, either proteins or RNA, across the nuclear envelope. Although there is currently no evidence that hsp70-related proteins perform this function, polypeptides of ~70 kD molecular mass that specifically bind nuclear localization signals have been identified in liver (Adam et al., 1989; Yamasaki et al., 1989) and yeast (Silver et al., 1989). In addition, hsp70-related polypeptides seem to be involved in protein transport into mitochondria and the ER (Chirico et al., 1988; Deshaies et al., 1988). Although signal-mediated protein uptake into the nucleus involves different mechanisms, these findings support the general hypothesis that B3 and B4 might be involved in transport.

Alternatively, the recycling property of hsp70-related polypeptides could be unique to the oocyte. Stage six oocytes are actively involved with the synthesis and storage of rRNA (LaMarca et al., 1973). The localization of hsp70 in the nucleus and nucleoli under stress conditions might be re-

quired for the recovery of nucleolar function (Pelham, 1984; Lewis and Pelham, 1985). It is conceivable that the heat shock cognates, B3 and B4, are involved in normal nucleolar processes in the oocyte, and facilitate increased rRNA production. Investigations are ongoing to determine the function of B3 and B4 in the oocyte, which might suggest a general function of recycling heat shock cognates.

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