In Vitro Transport of a Fluorescent Nuclear Protein and Exclusion of Non-Nuclear Proteins

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Abstract. An in vitro system was developed that provides a quick microscopic assay for nuclear transport. The assay uses an extract of *Xenopus* eggs, normal or synthetic nuclei, and a fluorescently labeled nuclear protein, nucleoplasmin. This in vitro system accurately mimics in vivo nuclear transport, both in exclusivity and in the amount of accumulation observed (up to 17-fold). Selective accumulation of fluorescent nucleoplasmin is observed microscopically within 30 min with rat liver nuclei, *Xenopus* embryonic nuclei, regrown *Xenopus* sperm nuclei, or nuclei reconstituted in vitro from bacteriophage lambda DNA. This transport requires the signal domain of nucleoplasmin. Furthermore, the ability of nuclei to accumulate nucleoplasmin directly correlates with their ability to exclude the fluorescent non-nuclear proteins, FITC-immunoglobulin and phycoerythrin.

An active transport model would predict that nuclear transport be temperature- and energy-dependent and that inhibition of transport by either low temperature or energy depletion would be reversible. Both predictions were confirmed in our system. Nucleoplasmin accumulation increases with temperature, while the protein is completely excluded at 0°C. The effects of low temperature are reversible. As found for ^{125}I-labeled nucleoplasmin (Newmeyer, D. D., J. M. Lucocq, T. R. Bürglin, and E. M. De Robertis, 1986, *EMBO (Eur. Mol. Biol. Organ.) J.*, 5:501–510), transport of fluorescent nucleoplasmin is inhibited by ATP depletion. This effect is reversed by later ATP addition. Under ATP-depleted conditions non-nuclear proteins continue to be excluded. These results argue for a direct role of ATP in transport rather than for a simple role in preserving envelope integrity.

In a first step towards defining the minimum requirements for a transport medium, egg extracts were depleted of membrane vesicles. Membrane-depleted extracts neither support transport nor maintain the integrity of the nuclear envelope.

In eukaryotic cells, the nuclear envelope serves as a semipermeable barrier segregating the genomic DNA from the cytoplasm. In the electron microscope, the nuclear envelope appears as two membrane bilayers separated by a perinuclear space. The inner nuclear membrane is lined by a polymeric proteinaceous layer, the lamina, which is composed of one or more lamin proteins (Aaronson and Blobel, 1974; Gerace and Blobel, 1980; Franke et al., 1981). Traversing the inner and outer membranes are numerous channels, the nuclear pores, which link the interior of the nucleus to the cytoplasm. Protein and RNA molecules enter and exit the nucleus through the nuclear pores (Stevens and Swift, 1966; Feldherr et al., 1984). It appears that selective regulation of the flow of materials through the pores is responsible for the distinctly different biochemical composition of the nucleoplasm and cytoplasm (Paine and Horowitz, 1980; De Robertis, 1983; Dingwall, 1985). There is evidence that nucleocytoplasmic exchange may also play an important role in regulating cellular functions during heat shock (Velasquez and Lindquist, 1984) and in response to cyclic AMP (Nigg et al., 1985).

Molecules with a diameter <90–110 Å diffuse freely through the pore, equilibrating between the nucleoplasm and cytoplasm (Paine et al., 1975; Lang et al., 1986), while molecules >90–110 Å do not enter the nucleus by passive diffusion. It has been suggested that facilitated diffusion and intranuclear binding could play a role in the selective accumulation of some large nuclear proteins (Feldherr and Ogburn, 1980; De Robertis, 1983; Einck and Bustin, 1984). Alternately, an active transport mechanism has been proposed for the transport of larger nuclear proteins (Dingwall et al., 1982; Feldherr et al., 1983 and 1984). Both mechanisms would allow transport to be selective and would result in a substantial accumulation of the appropriate proteins within the nucleus. The finding that ATP is required for transport of one nuclear protein (Newmeyer et al., 1986) supports an active transport mechanism, but does not preclude the existence of intranuclear binding for other proteins.

Several proteins that accumulate within the nucleus have been shown to contain one or more polypeptide sequences that are required for nuclear transport (e.g., Hall et al., 1984; Kalderon et al., 1984a and b). These sequences appear in principle to function like the signal sequences that specifically target proteins into the endoplasmic reticulum, mito-

chondria, or chloroplasts. Unlike mitochondrial targeting sequences, however, nuclear signal sequences are not removed during transport but remain part of the mature protein. Since most nuclear proteins are released to the cytoplasm at mitosis, a permanent signal sequence allows a nuclear protein to enter the nucleus not only immediately after its translation, but also during each interphase of the cell cycle.

Much of the current knowledge of nuclear transport has been obtained from studies of the *Xenopus* nuclear protein, nucleoplasmin. Within the oocyte, nucleoplasmin exists as a pentamer with a molecular mass of 165,000 D, composed of identical 33,000-D subunits (Dingwall et al., 1982). Nucleoplasmin constitutes 10% of the total nuclear protein of the oocyte (Mills et al., 1980; Krohne and Franke, 1980). Dingwall et al. (1982) have shown that the COOH-terminal third of each subunit of nucleoplasmin contains the signal domain for nuclear transport, while Feldherr et al. (1984) have shown that nucleoplasmin gold complexes can enter the nucleus through the pores.

To study nuclear transport we have developed an in vitro system that maintains nuclei in a functionally active state and permits experimental manipulation of transport. This system combines exogenously added nuclei with *Xenopus* egg extracts (Newport and Forbes, 1985; Newmeyer et al., 1986). Our choice of egg extracts as the medium for studying nuclear transport stemmed from the observation that all the structural components of nuclei (except DNA) are stored in the *Xenopus* egg in large amounts. When linear bacteriophage DNA was injected into *Xenopus* eggs, the DNA was found to assemble into structures comparable to normal eukaryotic nuclei by ultrastructural criteria and in their response to cell cycle modulators (Forbes et al., 1983). The reconstituted nuclei were shown to possess a nuclear lamina, nuclear pores, and an envelope consisting of two membrane bilayers (Forbes et al., 1983). Such reconstituted nuclei were also found to be active with respect to selective nuclear transport, since they contain small nuclear RNP particles, as judged by their staining with anti-small nuclear RNP antibodies (Newport and Forbes, 1985).

Nuclear reconstitution has since been shown to occur in vitro when bacteriophage DNA is added to a *Xenopus* egg extract (Forbes et al., 1983; Newport and Forbes, 1985; Newport et al., 1985; Newmeyer et al., 1986; Newport, J., manuscript submitted for publication). In vitro extracts are prepared from activated *Xenopus* eggs and are capable of reconstituting intact nuclei around added DNA. An ATP regenerating system enhances both reconstitution and the stability of the nuclei. A similar extract from *Rana pipiens* eggs has also been found (Lohka and Masui, 1983 and 1984) to be very efficient at assembling nuclear envelopes complete with nuclear pores around demembranated sperm nuclei. Similarly, an extract of cultured cells was shown to be capable of assembling nuclear structures around mitotic chromosomes (Burke and Gerace, 1986). In preliminary experiments, it was found that nuclei reconstituted in vitro appeared to transport labeled small nuclear RNPs but not tRNA molecules into the nucleus (Newport and Forbes, 1985). Recently, Newmeyer et al. (1986) have shown that nuclei assembled in vitro are capable of importing ^{125}I-labeled nucleoplasmin in an ATP-dependent manner. With these extracts, it should be possible to manipulate the conditions under which transport occurs to determine: (*a*) the optimal conditions for transport and (*b*) the events involved.

In this report, we describe results using a modified in vitro system that allows us to assay nuclear transport conveniently and quickly. To circumvent problems of nuclear heterogeneity and to shorten previous assays, we have developed an assay system that uses an extract of high efficiency, normal eukaryotic nuclei of uniform character, and a fluorescent transport substrate, rhodamine-labeled nucleoplasmin. With this system, transport of nucleoplasmin into nuclei is observed visually within 30 min. Selective nuclear accumulation of fluorescent nucleoplasmin occurs with nuclei derived from both homologous and heterologous species, as well as with synthetic nuclei formed using bacteriophage DNA. The in vitro fluorescence transport assay shows a strict correlation between nuclear envelope integrity and transport function. This implies that accumulation of nucleoplasmin does not involve intranuclear binding and supports an active role for the nuclear envelope in nucleoplasmin transport. We have further determined, by modifying both the extract and the assay conditions, a number of parameters that affect nuclear transport in our system and have tested an inhibitor of dextran influx into the nucleus for an effect on nuclear protein transport.

Materials and Methods

Materials

Tetramethylrhodamine isothiocyanate (TRITC),[1] hexokinase, apyrase (grade VIII), and L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin were obtained from Sigma Chemical Co. (St. Louis, MO). R-phycoerythrin was obtained from Molecular Probes, Inc. (Junction City, OR). FITC-concanavalin A (Con A) was purchased from Polysciences, Inc. (Warrington, PA) and rabbit anti-mouse FITC-immunoglobulin from Cappel Laboratories (Cochranville, PA).

Preparation of Nuclei

Rat liver nuclei were prepared essentially by the method of Blobel and Potter (1966) with modifications as described in Newport (manuscript submitted for publication). Aliquots of isolated rat liver nuclei were stored frozen at −70°C in the same buffer (with the addition of 250 mM sucrose) at a final concentration of 1–5 × 10^5 nuclei/μl.

Demembranated sperm nuclei were prepared by the method of Lohka and Masui (1983) and stored frozen at −70°C at a concentration of 1–4 × 10^5 nuclei/μl.

Xenopus embryonic nuclei were isolated from 9-h-old embryos. These were homogenized and the lysed embryo extract centrifuged in a clinical centrifuge to remove the yolk granules. The majority of embryonic nuclei were not removed by this centrifugation and could be examined for transport.

Preparation of Rhodamine-labeled Nucleoplasmin and Nucleoplasmin Core

Nucleoplasmin was isolated by a modification of the method of Dingwall et al. (1982), as described in Newmeyer et al. (1986). For conjugation of rhodamine to nucleoplasmin, TRITC was dissolved in dimethylsulfoxide to a concentration of 1 mg/ml. One-tenth volume of this was added to a solution containing 1 mg/ml nucleoplasmin in 0.1 M sodium carbonate buffer (pH 9), and 50 mM NaCl. In different conjugations, the mixture was allowed to react either for 1 h at ambient temperature or for 3 h at ambient temperature and, subsequently, overnight at 4°C. The free rhodamine isothiocyanate was removed and the protein exchanged into 50 mM Tris-HCl, pH 7.5, 50 mM NaCl by passage through a 1-ml column (Bio-gel P10; Bio-Rad Laboratories, Richmond, CA) equilibrated in this buffer. SDS-

1. *Abbreviation used in this paper*: TRITC, tetramethylrhodamine isothiocyanate.

PAGE on fractions from the column followed by visualization of the protein bands on an ultraviolet light box were done to check for labeling of the protein and separation from free label (Fig. 5).

For isolation of nucleoplasmin core protein lacking the signal sequence, a modification of the procedure of Feldherr et al. (1984) was used. Trypsin (grade VIII) was added to TRITC-labeled nucleoplasmin that removes the terminal third of each nucleoplasmin monomer, leaving a pentameric core of ~95,000 kD. The core was separated from the degradation products and trypsin by mixing the digest with 100 μl of DEAE-Sephacel in a microfuge tube and incubating for 15 min. The mixture was then loaded into a 200-μl disposable pipette tip plugged with glass wool. This microcolumn was washed with 50 mM Tris-HCl (pH 7.5), and 50 mM NaCl, before the nucleoplasmin core was eluted with 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5, according to Feldherr et al. (1984).

Preparation of Xenopus Egg Extracts

Extracts were prepared essentially by the method summarized in Newport and Forbes (1985) and presented in detail in Newport (manuscript submitted for publication). The jelly coats of *Xenopus* eggs were removed by incubation in 2% cysteine, pH 8, for 5 min. The eggs were activated with calcium ionophore A23187. Packed activated eggs were lysed in a buffer containing 250 mM sucrose, 50 mM KCl, 1 mM DTT, 2.5 mM MgCl, 0.02 mg/ml cycloheximide, and 0.005 mg/ml cytochalasin B by centrifugation for 5 min in a microfuge. The cleared extract bands, during centrifugation, between the pigment granule-yolk pellet and the lipids that float. The extract was withdrawn from the tube and recentrifuged in a microfuge for 5 min to clear the extract of remnants of pigment granules, etc. The extract was used immediately or within several hours on ice; it could be frozen in aliquots in liquid N_2 and stored at −70°C for later use, although frozen extract resulted in a smaller fraction of nuclei active for transport.

Nucleoplasmin Transport Assay

For the transport assay, nuclei (1 μl at 0.5–3 × 10^5/μl) or DNA (1 μl at 125 μg/ml) were added to 20 μl of egg extract containing 1–2.75 mM ATP, 9 mM creatine phosphate, and 100 U/μl creatine kinase. For assays with rat liver nuclei, the mixture was allowed to incubate for 30 min before addition of 1 μl of TRITC-labeled nucleoplasmin (final concentration, 12–15 ng/μl). Aliquots were withdrawn at various times for observation of the level of TRITC-nucleoplasmin in the nuclei compared with that present in the external egg extract. For microscopic observation, 4 μl of extract was applied to a slide and mixed with 0.5 μl of 37% formaldehyde and 0.5 μl of 10 μg/ml bisbenzimide DNA dye (Hoechst 33258; Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, CA) before application of a coverslip. For experiments with demembranated sperm nuclei or nuclei reconstituted from bacteriophage DNA, the nuclei were allowed to form (30 min–4 h) before the labeled nucleoplasmin was added.

The protocol for testing the effect of the temperature of incubation is described in the text. For ATP depletion experiments, the procedure of Newmeyer et al. (1986) was followed. Rat liver nuclei were allowed to incubate for 30 min in egg extract. At this time, apyrase (grade VIII; Sigma Chemical Co.) was added to a final concentration of 100 U/ml and the extract plus nuclei incubated for 30 min more at ambient temperature. One-twentieth volume of TRITC-labeled nucleoplasmin was then added and accumulation assayed 30 min later. To demonstrate that the inhibition of transport resulting from ATP depletion was reversible, it was necessary first to omit the ATP-regenerating system and to use hexokinase (100 U/ml) and glucose (10 mM) to deplete ATP. The standard ATP regeneration system (except that 20 mM phosphocreatine was used) was then added after 1 h along with TRITC-nucleoplasmin.

To prepare an egg extract depleted of membranous vesicles, 180 μl of fresh egg extract was subjected to ultracentrifugation at 100,000 g for 30 min in a cooled airfuge (Beckman Instruments, Inc., Fullerton, CA). The supernatant of this centrifugation was carefully removed and used as described in the text.

Fluorescence Microscopy and Quantitation of Accumulation

All observations were made using a Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) fitted for fluorescence microscopy with the exciter-barrier reflector combinations for the DNA dye, bisbenzimide, for fluorescein, and for rhodamine. Observations were made immediately after sample preparation.

To quantitate the level of accumulation of TRITC-labeled nucleoplasmin, individual nuclei that had accumulated nucleoplasmin were photographed using Kodak Tri X-Pan film. The film was developed and each negative of a nucleus scanned with a scanning densitometer (model 1650; Bio-Rad Laboratories, Richmond, CA). The densitometer was zeroed to the absorbance present in the space between negatives. A chart recorder was used to record the absorbance over the nucleus and over the media external to the nucleus. By calculating the ratio of the value found for the accumulating nucleus to the value found for the background fluorescence of the egg extract, an approximate value for accumulation was derived. Such values were corrected for the slight dilution (1.25-fold) of the external solution caused by addition of fixative and DNA dye. Final values for accumulation ranged up to 17.2-fold.

Con A Staining and Test for Inhibition

To assay binding of FITC-labeled Con A to nuclei in egg extracts, one of two procedures was followed. In one, FITC-Con A (0.1 mg/ml) was added to the extract containing nuclei and the nuclei observed microscopically 30 min later. In the second, FITC-Con A (0.5 μl of 1 mg/ml) was added to a slide with a 5-μl sample of nuclei in extract and the mixture examined immediately.

To test the effect of Con A on nucleoplasmin transport, FITC-lectin or unlabeled Con A (final concentration, 0.1 mg/ml) was added to 20 μl of extract-containing rat liver nuclei (1–5 × 10^5) that had been incubated in extract for 20 min. After a 5-min preincubation with Con A, 1 μl of TRITC-nucleoplasmin was added (final concentration, 15 ng/μl). Accumulation was assayed microscopically 20–30 min later. In parallel control incubations an equivalent volume of PBS was added instead of FITC-Con A.

Results

Nuclear and Cytoplasmic Assay Components

Two extracts prepared from activated *Xenopus* eggs have previously been described as able to package added DNA (Forbes et al., 1983; Newport and Forbes, 1985; Newport et al., 1985; Newmeyer et al., 1986; Newport, J., manuscript submitted for publication) or demembranated sperm nuclei (Lohka and Masui, 1983 and 1984) into structures resembling eukaryotic nuclei at the electron microscopic level. We have used one of these extracts to design a quick in vitro assay system for nuclear transport. The extract used was prepared in the presence of 20 μg/ml cycloheximide (to block the cell cycle at the end of S phase; Miake-Lye et al., 1983) and 5 μg/ml cytochalasin B (to disrupt actin filaments) (Newport and Forbes, 1985; Newport, J., manuscript submitted for publication). This extract gives consistently good formation of synthetic nuclei from added bacteriophage DNA. Phage DNA is assembled within 2 h of addition into membrane-enclosed spheres that stain in a uniform manner with the fluorescent DNA dye, bisbenzimide. By 4 h, the synthetic nuclei swell to become large spheres (Fig. 1*a*), similar in staining pattern to normal eukaryotic nuclei. When demembranated sperm nuclei were added to our extract, the condensed sperm DNA decondensed, acquired a nuclear envelope, and swelled to a large size as first described by Lohka and Masui (1983). Thus, the egg extract contains all the nuclear components, with the exception of DNA, necessary for formation of synthetic nuclei.

To test the effect of the egg extract on the structure and stability of pre-existing eukaryotic nuclei, isolated rat liver nuclei were added to the extract and examined for morphology by staining with bisbenzimide. The majority of rat liver nuclei, when incubated in the extract for up to 90 min, retained their general shape and size. However, phase-contrast microscopy indicated that the nuclear membranes had grown: many nuclei after 30 min in the extract contained a

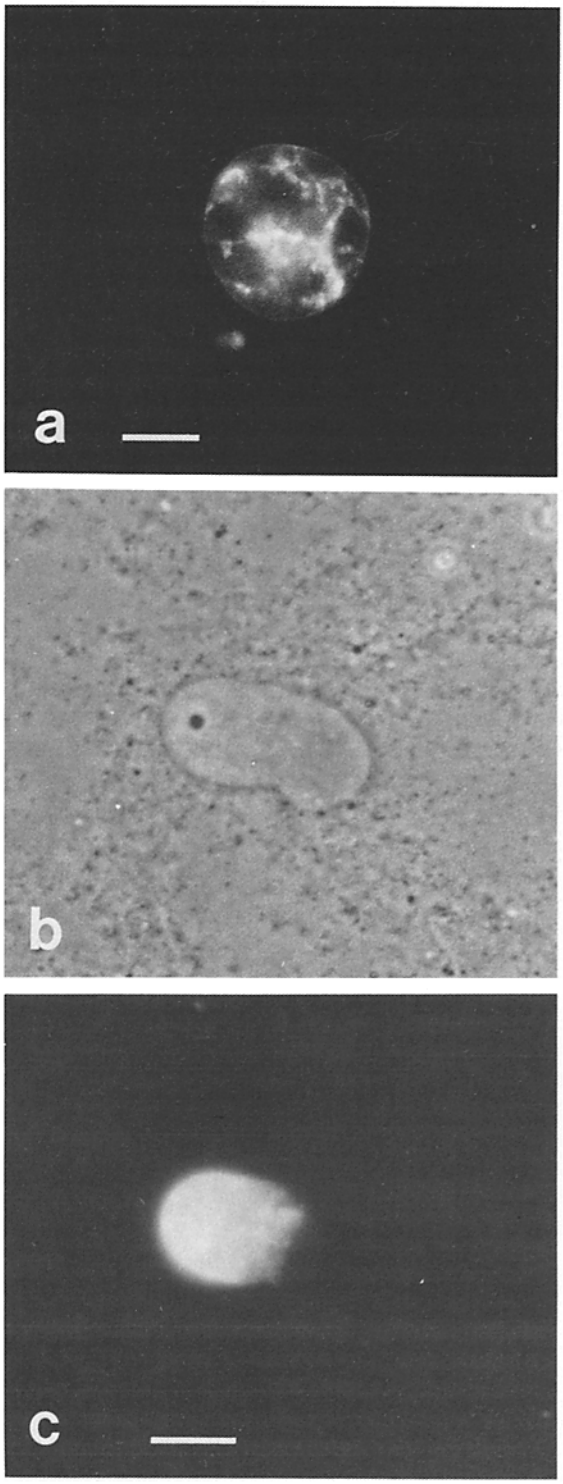

Figure 1. (*a*) A synthetic nucleus reconstituted in vitro. A typical nucleus containing bacteriophage lambda DNA is shown. Reconstitution was as described in Materials and Methods. (*b* and *c*) Rat liver nuclei undergo nuclear envelope growth when incubated in extract. A phase-contrast micrograph (*b*) and a fluorescence micrograph of DNA stained with bisbenzimide (*c*) of one such nucleus is shown. In the majority of nuclei observed in this manner, the DNA remained connected to the original nuclear envelope. Bar, 10 μm.

contiguous extension of envelope equal to or larger in size than the original nucleus (Fig. 1, *b* and *c*). Fig. 2 shows three rat liver nuclei that have undergone nuclear membrane growth, as visualized with a fluorescent molecule that is unable to enter the nucleus (Fig. 2 *b*). The nuclear membrane extension is presumably due to incorporation of *Xenopus* nuclear membrane into the rat liver nuclear membrane. We also observed nuclei that contained either no additional membrane or two such membranous extensions or had swollen uniformly into large spheres. Such changes in nuclear membrane size resemble those seen in vivo in the nuclei of embryos incubated in cycloheximide (Forbes et al., 1983). These morphological results, coupled with the results of staining with a fluorescent lectin (see below), lead us to conclude that the egg extract maintains the existing nuclear envelopes of rat liver nuclei in an intact state. Furthermore, the extract can integrate new membrane components into the pre-existing nuclear envelope, resulting in membrane growth and a hybrid nuclear envelope.

A Rapid Microscopic Assay for Nuclear Protein Transport

To assay authentic accumulation of a nuclear protein, it was first necessary to verify that non-nuclear proteins are excluded by nuclei under the conditions of our assay. We used a nuclear protein, nucleoplasmin, as a positive nuclear transport substrate, and two non-nuclear proteins, FITC-immunoglobulin and phycoerythrin, as negative transport substrates. All three proteins are large (>150,000 D) and are thus incapable of passive diffusion into the nucleus (Bonner, 1975; Paine et al., 1975; Feldherr and Ogburn, 1980; Einck and Bustin, 1984). The integrity of a rat liver nucleus would be reflected in its ability to exclude FITC-immunoglobulin or the fluorescent algal protein, phycoerythrin, while the capacity of a nucleus for nuclear transport would be indicated by accumulation of nucleoplasmin. We found that ~50% of the nuclei incubated in the egg extract for 20 min excluded FITC-immunoglobulin and phycoerythrin. The exclusion of phycoerythrin from representative liver nuclei and their associated nuclear membrane blebs is shown in Fig. 2. The nuclei appear dark against the background of phycoerythrin fluorescence. Nuclei that did not exclude these proteins were usually damaged, as judged by phase-contrast microscopy (a discontinuous nuclear membrane), or by DNA staining pattern (i.e., DNA outside the membrane).

To observe nuclear transport in a microscopic assay, nucleoplasmin was first fluorescently labeled with TRITC. For the assay, rat liver nuclei ($0.5–3 \times 10^5$) were added to 20 μl of egg extract, supplemented with an ATP regenerating system (which improved nuclear morphology noticeably), and incubated for 30 min at 22°C to allow equilibration with the extract and possible healing of small membrane perforations. After 30 min, rhodamine-labeled nucleoplasmin was added. Aliquots were withdrawn at different times and the nuclei assayed for accumulation of TRITC-nucleoplasmin by fluorescence microscopy. Accumulation of rhodamine-labeled nucleoplasmin within the nuclei was observed by 15 min and appeared maximal by 30–45 min (Fig. 3 *a*). Approximately 50% of the rat liver nuclei were seen to accumulate nucleoplasmin, consistent with the fraction of nuclei that excluded phycoerythrin. In experiments where both FITC-im-

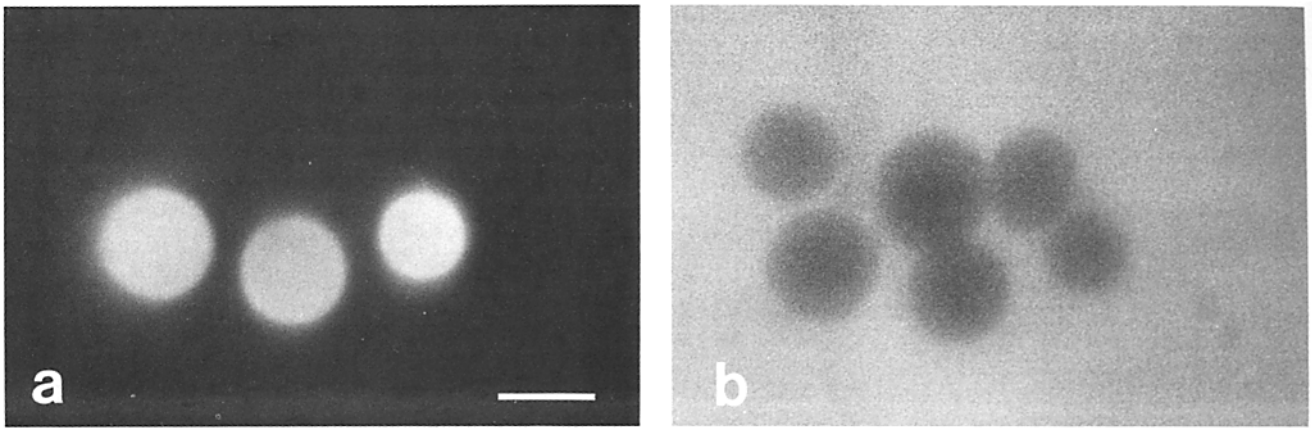

Figure 2. Many nuclei incubated in an egg extract undergo membrane growth and are intact, inasmuch as they exclude large proteins. Rat liver nuclei were incubated for 30 min in egg extract in the presence of the large fluorescent protein, phycoerythrin, then fixed, stained with the DNA dye, bisbenzimide, and examined in the fluorescence microscope using filter combinations appropriate for each fluorochrome. (All three blebs of the nuclei shown are connected to their respective nuclei, although different focal planes cause that of the nucleus on the left to appear disconnected.) (*a*) DNA fluorescence; (*b*) phycoerythrin fluorescence. Bar, 10 μm.

munoglobulin and TRITC–nucleoplasmin were present, all those nuclei that accumulated the latter protein excluded the former (Fig. 3, *a* and *b*).

The presence of nucleoplasmin in the nucleus was determined to be accumulation due to nuclear transport rather than DNA binding, since both the rat liver nucleus and the membranous bleb lacking DNA showed equally high and uniform rhodamine fluorescence (Fig. 4 *c*). Moreover, no binding of rhodamine-labeled nucleoplasmin to DNA was observed in damaged nuclei. The fluorescence ranged up to 17-fold higher than the background level of fluorescence (see below for quantitation). Thus, nuclear transport of nucleoplasmin occurs in these extracts and can be quickly and easily assayed.

During the course of these experiments, it was found that FITC–Con A stained only the nuclear envelopes of damaged rat liver nuclei (Fig. 4 *b*). This provided another visual assay that distinguished intact nuclei from damaged nuclei unable to accumulate nucleoplasmin. Therefore, staining with FITC–Con A was done concurrently with the TRITC–nucleoplasmin transport assay. Of the two nuclei shown in Fig. 4, the upper nucleus is damaged, as judged by a smeared DNA staining pattern (Fig. 4 *a*) and the presence of FITC–Con A staining (Fig. 4 *b*). The lower nucleus is intact, as judged by the absence of FITC–Con A staining (Fig. 4 *b*). This nucleus had accumulated TRITC-labeled nucleoplasmin (Fig. 4 *c*), and the upper damaged nucleus had not. From such double staining we found that all nuclei that accumulated fluorescent nucleoplasmin failed to stain with FITC–Con A. We conclude that nuclei that accumulate nucleoplasmin do not have gaps in their nuclear membrane that allow access and binding of FITC–Con A to the perinuclear space. This result is consistent with the accumulation of nucleoplasmin being the result of authentic transport through the nuclear pores of intact nuclear envelopes.

To show that transport in our system depends on the nuclear localization signal of nucleoplasmin, TRITC–nucleoplasmin was digested with trypsin to produce a fluorescent core protein lacking the signal domain (Dingwall et al., 1982) (Fig. 5, lane 2). When the TRITC-nucleoplasmin core

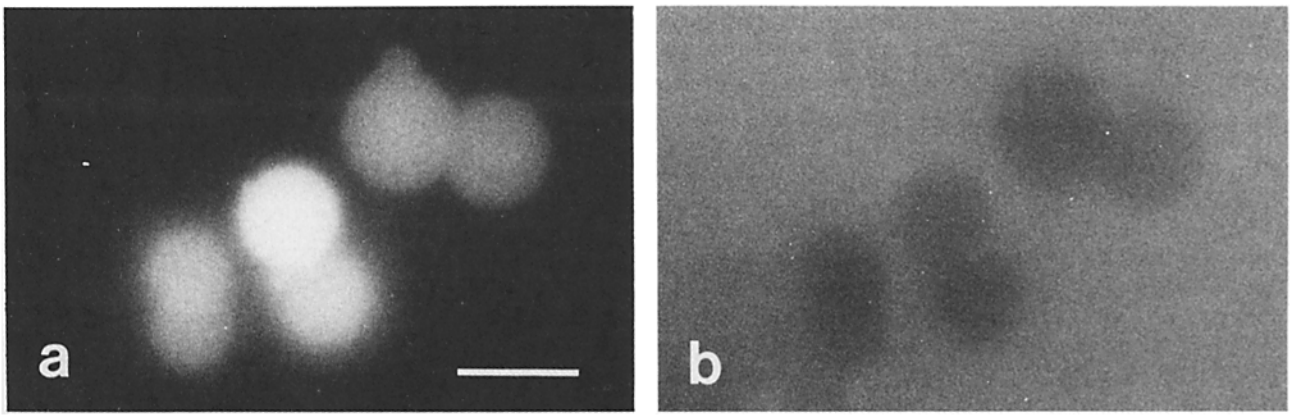

Figure 3. Simultaneous accumulation of a nuclear protein and exclusion of a non-nuclear protein. Rat liver nuclei were incubated in egg extract 30 min before the addition of TRITC-labeled nucleoplasmin and FITC-labeled immunoglobulin. After a further 30-min incubation, an aliquot was placed on a slide and examined. (*a*) Three rat liver nuclei and their associated membranous blebs can be seen to be accumulating TRITC–nucleoplasmin. (*b*) The same nuclei can be seen to be intact by their exclusion of FITC–immunoglobulin. Bar, 10 μm.

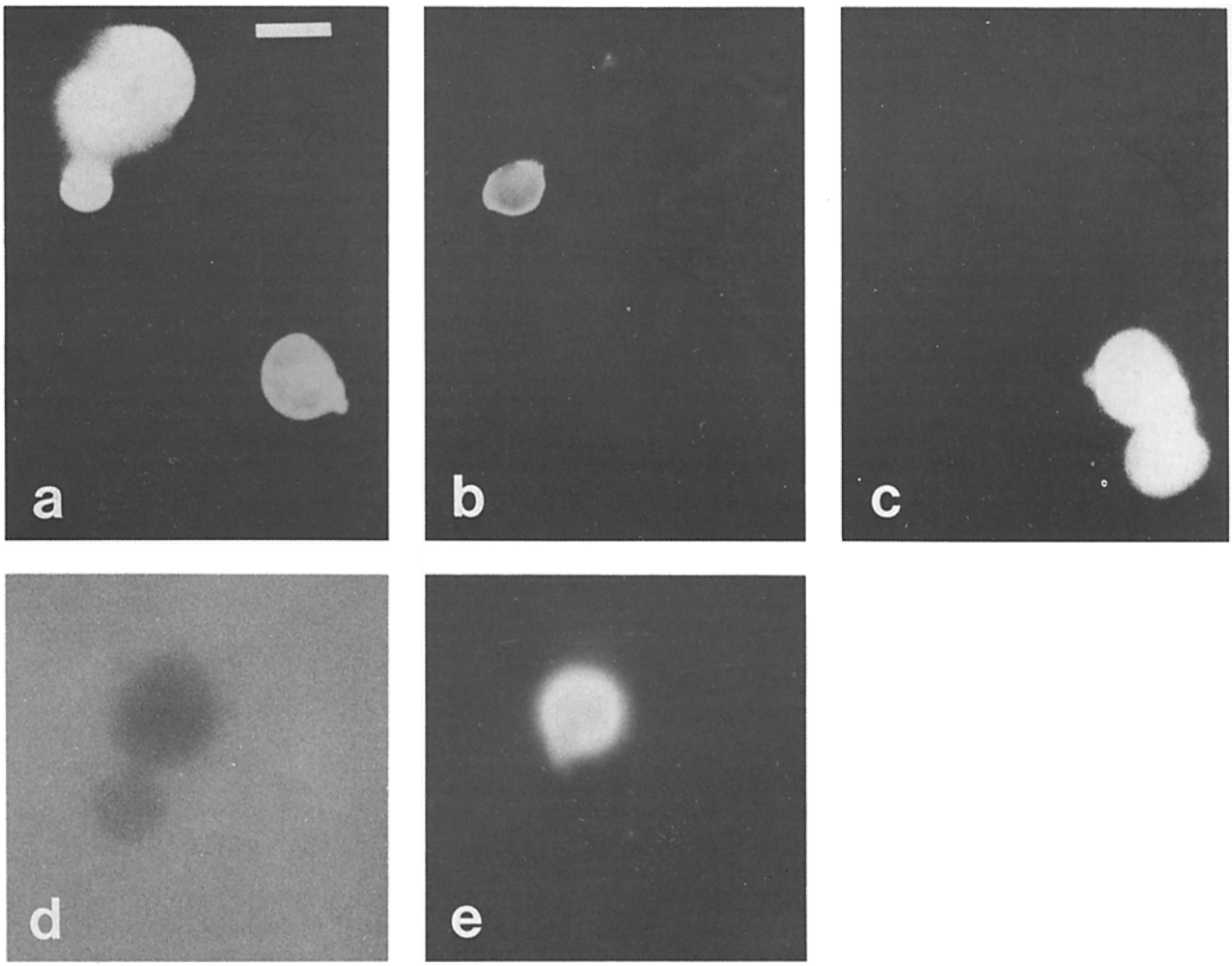

Figure 4. (*a–c*) Correlation between the ability of a nucleus to transport nucleoplasmin and its lack of staining by Con A. Rat liver nuclei were incubated in egg extract for 20 min. FITC–Con A and TRITC–nucleoplasmin were then added for 30 min. (*a*) Bisbenzimide (DNA) fluorescence; (*b*) FITC–Con A fluorescence; (*c*) TRITC–nucleoplasmin fluorescence. (It should be noted that a fraction of the nuclei neither accumulated nucleoplasmin nor were stained with FITC–Con A. In an experiment in which 51% of the nuclei excluded phycoerythrin and 50% accumulated nucleoplasmin, 10% of those not accumulating nucleoplasmin were not stained with FITC–Con A.) (*d* and *e*) The trypsin-resistant core of the nucleoplasmin pentamer is not accumulated by nuclei in egg extracts and is excluded from a fraction of intact nuclei. (*d*) TRITC–nucleoplasmin core fluorescence; (*e*) bisbenzimide (DNA) fluorescence. Bar, 10 μm.

was added to rat liver nuclei in extract, it did not accumulate and was in fact excluded from some intact nuclei (Fig. 4, *d* and *e*). This indicates that the transport we observe with intact nucleoplasmin does require the signal sequence.

Both Normal and Synthetic Nuclei Accumulate Fluorescent Nucleoplasmin

Nuclear transport of fluorescent nucleoplasmin was also observed in homologous *Xenopus laevis* embryonic nuclei incubated in an embryonic extract. Embryonic nuclei were obtained and tested as follows. Embryos were grown to the pigmented crescent stage (early gastrula), lysed, and the yolk removed by low speed centrifugation, leaving a mixture of embryonic cytoplasm and nuclei. TRITC–nucleoplasmin was added to this mixture and transport assayed microscopically. 70–90% of the embryonic nuclei were capable of transport and accumulated nucleoplasmin to a high extent (Fig. 6, *a* and *b*). (In these nuclei, we also observed extremely bright staining of two intranuclear objects, which may be nucleoli or prenucleolar structures. The reason for this staining is unknown.)

Similarly, nuclear transport was observed with regrown *Xenopus* sperm nuclei. Demembranated sperm nuclei were added to an egg extract where they reacquired a nuclear envelope, decondensed their DNA and, within 20–30 min, swelled to sizes several times larger than rat liver nuclei. At 30 min, rhodamine-labeled nucleoplasmin was added. Nuclear fluorescence became apparent within 15 min and increased with time, reaching an apparent maximum at 30–45 min after nucleoplasmin addition. *Xenopus* sperm nuclei were more fragile than rat liver nuclei, perhaps due to their larger size. Assay aliquots had to be treated with care in order to visualize unbroken nuclei. However, up to 75% of the reformed sperm nuclei transported and accumulated TRITC–nucleoplasmin. One such sperm nucleus is shown in Fig. 6 *c*.

To test whether nuclei reconstituted from bacteriophage lambda DNA were able to transport and accumulate rhodamine-labeled nucleoplasmin in this assay, as previ-

ously reported for ^{125}I-labeled nucleoplasmin visualized by autoradiography (Newmeyer et al., 1986), phage DNA was added to the egg extract in the presence of an ATP regenerating system. The nuclear formation extract used here was almost uniformly successful (9/10 extracts) in producing large numbers of synthetic nuclei whose DNA stains with bisbenzimide in the manner shown in Fig. 1. For the transport assay, TRITC-labeled nucleoplasmin was added to the nuclei at an early stage (~120 min after the DNA was added) when the DNA stained in a uniform manner (as visualized with bisbenzimide) and was enclosed in a nuclear membrane (as visualized with phase-contrast microscopy). The synthetic nuclei accumulated nucleoplasmin (Fig. 6, lanes *e–g*) to levels similar to those seen with regrown sperm nuclei. When nucleoplasmin was added to synthetic nuclei that had reached the stage where the DNA appears to be attached primarily to the nuclear periphery, these nuclei were also seen to accumulate nucleoplasmin. In summary, four different kinds of nuclei, rat liver nuclei, *Xenopus* embryonic nuclei, regrown *Xenopus* sperm nuclei, and nuclei reconstituted from lambda DNA, were found capable of nuclear transport in vitro using the *Xenopus* extract described here.

Requirement for Membrane Vesicles

The transport extracts used in the above experiments were prepared freshly each day, as described in Materials and Methods. When extracts frozen in liquid nitrogen and stored at −70°C were substituted for fresh extract, rat liver nuclei accumulated nucleoplasmin to the usual degree, but fewer nuclei appeared capable of accumulation. One explanation is that a proportion of the membrane vesicles in the extract were damaged by freezing and thawing and were not available to heal minor damage of the rat liver nuclear membranes, healing which would normally occur in fresh extracts.

To test whether the presence of membrane vesicles in the transport-competent egg extract was essential for our system, the extract was subjected to an additional fractionation step. Centrifugation at 100,000 *g* for 30 min removed all visible membrane vesicles. (In agreement with the finding of Lohka and Masui [1984], ultracentrifuged extracts did not promote membrane reconstitution around demembranated sperm nuclei.) When rat liver nuclei were added to such extracts the nuclei did not acquire the characteristic membrane blebs, failed to accumulate rhodamine-labeled nucleoplasmin and, in fact, lysed. It appears that membrane vesicles are needed to maintain the integrity of the rat liver nuclei or to repair damage previously incurred during isolation. However, when an ultracentrifuged extract was used to dilute a standard extract to which rat liver nuclei had been added 30 min previously, dilution up to 32-fold with the vesicle-depleted extract did not interfere with transport of subsequently added TRITC–nucleoplasmin. Thus, preincubation of the rat liver nuclei with membrane-containing extract presumably repairs the nuclei and, once repaired, only a small amount of membrane in the extract is necessary for continued nuclear stability. At present we do not know whether nonmembranous components of the extract are also required for nuclear transport.

Temperature Affects Nuclear Protein Transport

We determined the effect of temperature on in vitro nuclear transport using both rat liver nuclei and sperm nuclei. Nuclei were preincubated in extract at 22°C for 30 min, then shifted to the assay temperature (0, 14, 22, 30, 37, or 40°C). After a 5-min incubation at the indicated temperatures, TRITC–nucleoplasmin was added and the extract maintained at the new temperature. 30 min later, aliquots were withdrawn, fixed with formaldehyde, observed under the fluorescence microscope, and photographed. The amount of nuclear accumulation was calculated as the ratio of the nuclear signal intensity to the background intensity, as measured by densitometric scanning of photographic negatives of the accumulating nuclei.

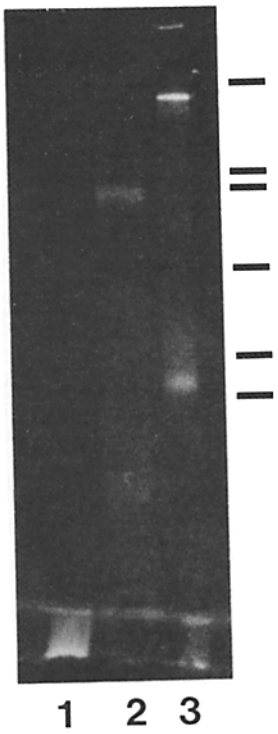

Figure 5. SDS-PAGE of TRITC–nucleoplasmin, TRITC–nucleoplasmin core, and free TRITC. Samples were mixed with SDS-mercaptoethanol sample buffer but not boiled; as a result, nucleoplasmin is seen here in both the monomeric and pentameric forms (Dingwall et al., 1982, Feldherr et al., 1984). Gel electrophoresis was performed as in Laemmli (1970). The protein bands were visualized on an ultraviolet light box and photographed. Bars at the right represent positions of the following molecular weight standards (*from top to bottom*): myosin heavy chain (205 kD), Beta-galactosidase (116 kD), phosphorylase b (97.4 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD). Lane *1*, free TRITC; lane *2*, TRITC–nucleoplasmin core; lane *3*, TRITC–nucleoplasmin.

In regrown sperm nuclei, nuclear accumulation of TRITC–nucleoplasmin varied with temperature, as shown in Fig. 7 *a*. Essentially no accumulation was seen at 0°C. As the temperature increased up to 30°C, the average amount of TRITC–nucleoplasmin accumulation increased. Above 30°C, however, accumulation dropped. The same temperature-dependent behavior was seen in three sets of experiments with regrown *Xenopus* sperm nuclei with accumulation being negligible at 0°C and increasing as the temperature was raised from 14 to 30°C.

A similar result was obtained with rat liver nuclei. At 0°C, nucleoplasmin was excluded from the nuclei. Good accumulation was observed at 22 and 30°C, less at 37°C (Fig. 7 *b*). Because many more nuclei were stained by FITC–Con A at the higher temperature (37°C), we surmise that the drop in accumulation observed at 37°C was at least partly due to nuclear damage. Nuclear fragility at high temperature may also account for the drop in accumulation observed with sperm nuclei. We conclude that transport of nucleoplasmin increases in both rat liver and *Xenopus* sperm nuclei as the temperature is increased to 30°C, and above 30°C nuclei appear increasingly unstable; transport does not occur at 0°C,

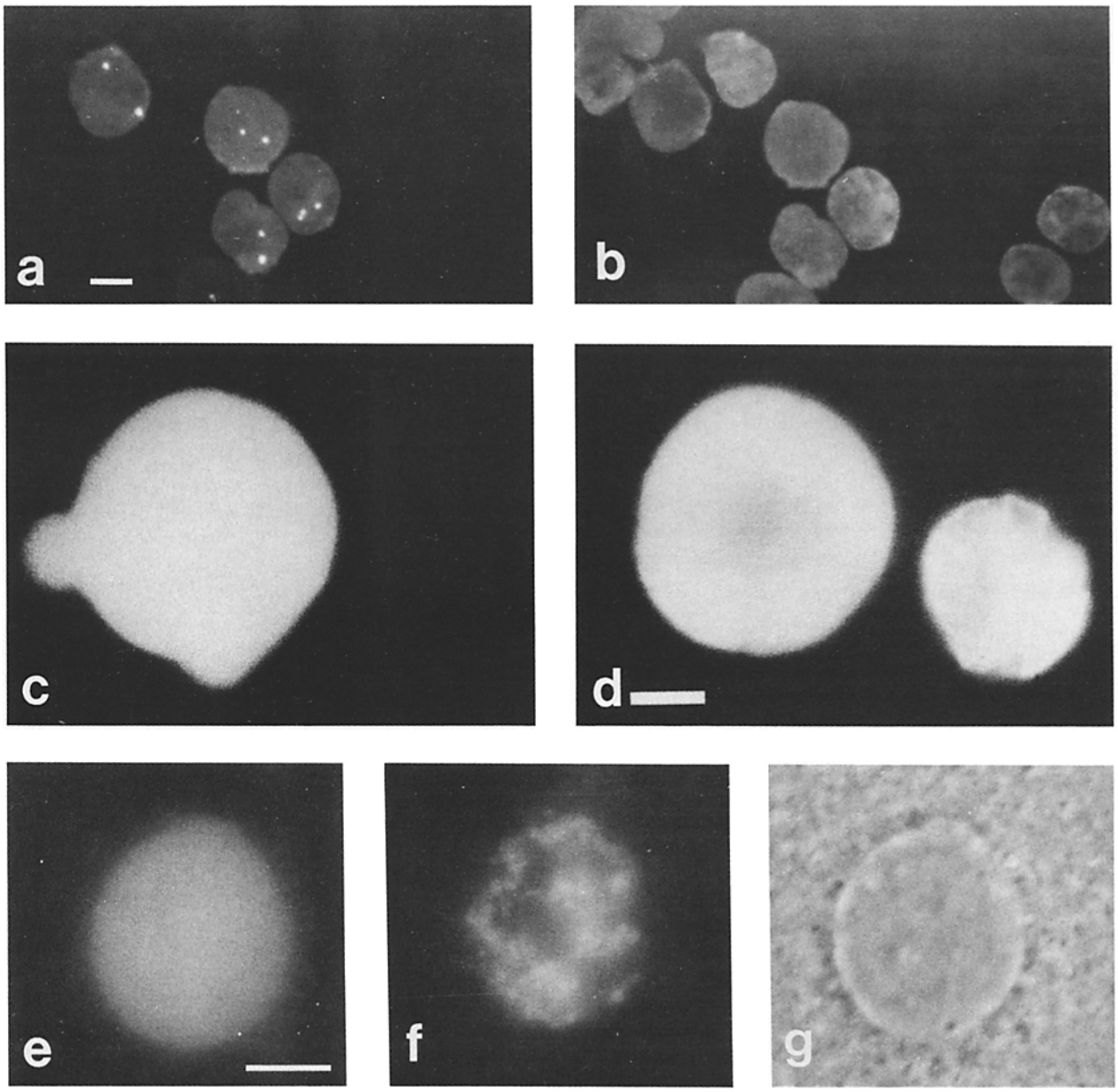

Figure 6. Nucleoplasmin transport into different types of nuclei. (*a* and *b*) *Xenopus* embryonic nuclei; (*c* and *d*) regrown *Xenopus* sperm nuclei; (*e–g*) a synthetic nucleus assembled in vitro from lambda DNA. (*a*, *c*, and *e*) TRITC–nucleoplasmin fluorescence; (*b*, *d*, and *f*) bisbenzimide (DNA) fluorescence; (*g*) phase contrast. Bar, 10 μm.

but is restored when the temperature is shifted to 22°C (not shown).

Inhibition of Nuclear Protein Transport by ATP Depletion Is Reversible

The experiments detailed above make it clear that the integrity of the nucleus is a prerequisite for accumulation of nucleoplasmin. In those experiments, an ATP regenerating system was added at the time of addition of nuclei or DNA. If, however, ATP was removed from the extracts by the addition of an exogenous ATPase, rat liver nuclei underwent chromatin condensation (Fig. 8 *b*) and were unable to accumulate nucleoplasmin (Fig. 8 *a*), as previously shown for synthetic nuclei (Newmeyer et al., 1986). The nuclear envelopes of these nuclei remained intact under ATP-depleted conditions, since the nuclei retained the ability to exclude phycoerythrin and FITC–immunoglobulin (not shown). We asked whether the effects of ATP depletion could be reversed by subsequent addition of an ATP regenerating system. Rat liver nuclei were preincubated for 30 min in extract from which the regenerating system had been omitted. To deplete ATP endogenous to the extract, hexokinase and glucose were added. After 1 h, the ATP-regenerating system was added along with TRITC–nucleoplasmin. Transport was assayed 30 min later with the fluorescence microscope. It was found that re-addition of ATP to the system both restored the ability of nuclei to accumulate nucleoplasmin and reversed the chromatin condensation that had occurred in the absence of ATP (Fig. 8, *c–f*). We conclude that ATP depletion causes no irre-

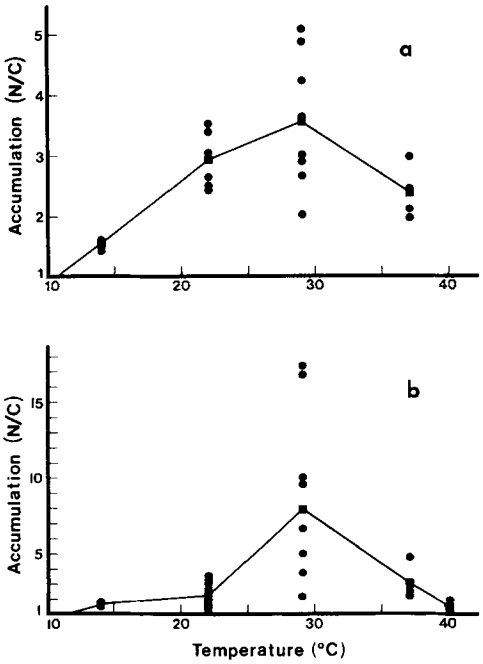

Figure 7. Temperature dependence of nucleoplasmin transport by regrown *Xenopus* sperm nuclei and rat liver nuclei in *Xenopus* egg extract. Solid circles represent nucleoplasmin accumulation ratios for individual nuclei, while solid squares are average values. Transport was quantitated as the ratio of nuclear to external (cytoplasmic) fluorescence (N/C) as described in Materials and Methods. (*a*) *Xenopus* sperm nuclei; (*b*) rat liver nuclei.

versible damage, either to the nuclear envelope or to the nuclear pores. This is consistent with a direct involvement of ATP in protein translocation into the nucleus.

The Lectin Con A Does Not Block Nuclear Protein Transport

Recently, Jiang and Schindler (1986) reported that addition of 0.1 mg/ml Con A to rat liver nuclei in buffer inhibited the influx of a 64-kD fluorescent dextran 20-fold. In that assay, ATP was not present. When ATP was added, the inhibition decreased to 2–10-fold. To test the effect of Con A on nucleoplasmin transport, we added FITC–Con A or unlabeled Con A to rat liver nuclei in egg extract before the addition of TRITC–nucleoplasmin. Accumulation was assayed microscopically 30 min after nucleoplasmin addition. As stated above, FITC–Con A stained only damaged nuclei and did not stain nuclei that had accumulated nucleoplasmin when it was added after nucleoplasmin accumulation. We concluded from those results that the space between the inner and outer membranes of nuclei active in nucleoplasmin transport was inaccessible to Con A. When Con A was added just before nucleoplasmin addition, no effect on accumulation was observed (Table I). Even with a prolonged preincubation, FITC–Con A did not stain nuclei accumulating nucleoplasmin.

Discussion

We have developed a rapid in vitro assay for nuclear transport using an extract from *Xenopus* eggs and a rhodamine-labeled nuclear protein, nucleoplasmin. We observe nuclear

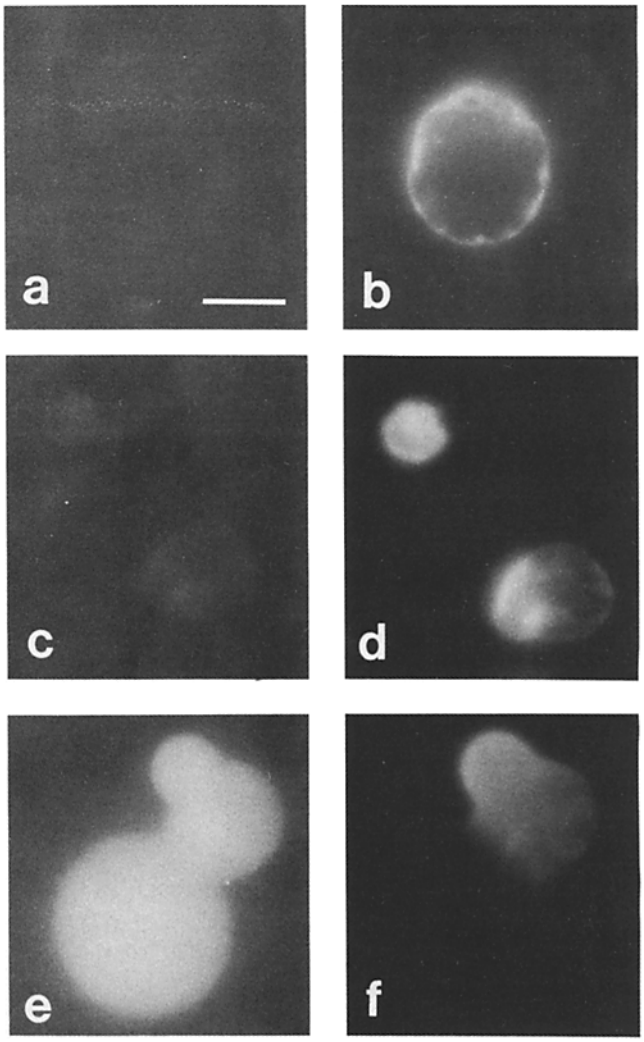

Figure 8. Inhibition of nuclear transport by ATP depletion is reversible. (*a* and *b*) Rat liver nuclei were incubated in egg extract for 30 min, at which time apyrase was added to a final concentration of 100 U/ml to deplete ATP. After 30 min, TRITC–nucleoplasmin was then added for a final 30-min incubation. No transport was observed. (*a*) TRITC–nucleoplasmin fluorescence; (*b*) bisbenzimide (DNA) fluorescence. (*c* and *d*) Rat liver nuclei were incubated in egg extract in the absence of an ATP regenerating system. Hexokinase and glucose were added to deplete ATP. After 1 h, TRITC–nucleoplasmin was added and transport assayed 30 min later. No transport was observed. (*c*) TRITC–nucleoplasmin fluorescence; (*d*) bisbenzimide (DNA) fluorescence. (*e* and *f*) Rat liver nuclei were depleted of ATP as in *c* and *d*. An ATP regenerating system was added at the time of TRITC–nucleoplasmin addition. The effects of ATP depletion can be seen to be reversed. (*e*) TRITC–nucleoplasmin fluorescence; (*f*) DNA fluorescence. Bar, 10 μm.

transport in vitro using purified nuclei from rat liver and *Xenopus* embryos, as well as synthetic nuclei assembled around demembranated *Xenopus* sperm nuclei or bacteriophage DNA. The rapidity of the assay has allowed us to study a number of parameters that affect nuclear transport. The results are summarized in Table I.

A number of observations indicate that this in vitro transport system is a faithful model for nuclear transport as it occurs in vivo. Large proteins that lack a nuclear targeting sequence (FITC–immunoglobulin, phycoerythrin) do not enter

Table I. Summary of the Conditions Affecting Nuclear Transport

Nuclei	Equilibration	Exclusion	Accumulation
Rat liver nuclei			
FITC-IgG		+	
Phycoerythrin		+	
TRITC-nucleoplasmin			
0°C		+	
22°C			+
30°C			+
0°C, then 22°C			+
+ Con A			+
+ apyrase	+		
+ hexokinase/glucose	+		
+ hexokinase/glucose, later ATP			+
TRITC-nucleoplasmin core		+	
Xenopus sperm nuclei			
TRITC-nucleoplasmin, 22°C			+
Xenopus embryonic nuclei			
TRITC-nucleoplasmin, 22°C			+
Synthetic nuclei			
TRITC-nucleoplasmin, 22°C			+

the nucleus in our in vitro system. On the other hand, TRITC-labeled nucleoplasmin that contains a signal sequence is not only transported into the nuclei, but also accumulates in the nuclei to a considerable extent (~17-fold). An TRITC-labeled nucleoplasmin core protein from which the signal sequence has been removed does not accumulate in nuclei. Transport of nucleoplasmin occurs in either freshly prepared or frozen extracts from activated *Xenopus* eggs and with either freshly isolated or frozen rat liver nuclei. ATP, as expected from previous results (Newmeyer et al., 1986), is required for accumulation of fluorescent nucleoplasmin. Nuclei in ATP-depleted extracts retain their ability to exclude non-nuclear proteins indicating that ATP is not required for envelope stability, but is required for nucleoplasmin transport. The amount of nucleoplasmin transported per nucleus in a fixed amount of time increases as the temperature is increased up to 30°C, but transport does not occur at 0°C, at which temperature nucleoplasmin is excluded from nuclei.

The egg extracts function well at maintaining the nuclear envelopes of added nuclei in a functional state. The exclusion of phycoerythrin and FITC-immunoglobulin indicates that the nuclear envelopes retain their selectivity in extracts. Moreover, the extracts support nuclear envelope growth. Nuclei with nuclear membranous extensions continue to exclude large non-nuclear molecules. Our observation of high levels of accumulation of nucleoplasmin and the finding that accumulation of nucleoplasmin is always correlated with the exclusion of large non-nuclear proteins argues that the envelopes are maintained by the extract in a functional state with respect to nuclear transport. Nuclei with damaged envelopes do not accumulate nucleoplasmin. Hence, unlike Feldherr and Ogburn (1980) who found that some nuclear proteins can accumulate in punctured *Xenopus* oocyte nuclei, we observe a requirement for nuclear envelope integrity.

From previous work it is clear that *Xenopus* nuclear envelopes can form around added DNA or around demembranated *Xenopus* sperm nuclei (Forbes et al., 1983; Lohka and Masui, 1983 and 1984; Newport and Forbes, 1985; Newport et al., 1985; Newmeyer et al., 1986). From the studies reported here with rat liver nuclei, it appears further that *Xenopus* egg extracts can promote formation of a hybrid rat–*Xenopus* nuclear envelope that is competent both to transport and to retain a *Xenopus* protein, nucleoplasmin.

To assess the relative efficiency of the transport observed with our in vitro fluorescence assay, we compared our data with the results obtained by Dingwall et al. (1982) for nuclear transport of nucleoplasmin in vivo. When these workers injected radiolabeled nucleoplasmin into the cytoplasm of *Xenopus* oocytes, dissected the oocytes into nuclear and cytoplasmic fractions 30 min later, and fractionated the labeled nucleoplasmin protein on gels, they found that nucleoplasmin was enriched in the nucleus. Assuming that the nuclear volume was 1/25th of the cytoplasmic volume, they estimated that nuclear accumulation was 35-fold within 30 min. If, however, the volume of yolk platelets in the cytoplasm had been taken into consideration in the calculation (the nucleus contains 12% of the volume of the yolk-free cytoplasm [Bonner, 1978]), the relative accumulation would be 12-fold. To compare accurately the transport observed in vitro with that in vivo, we must first correct for differences in at least three parameters: (*a*) the surface area/volume ratio of the nuclei, (*b*) the external unlabeled nucleoplasmin, which can compete with labeled nucleoplasmin for transport, and (*c*) the number of nuclear pores per mm^2 of nuclear envelope. The surface area/volume ratio of a rat liver nucleus is ~40 times that of an oocyte nucleus. If this were the only difference between the two nuclei, it would result in a 40-fold enhanced accumulation with rat liver nuclei compared with oocyte nuclei. The external unlabeled nucleoplasmin concentration, however, must also be considered. In the oocyte, essentially no unlabeled nucleoplasmin exists in the cytoplasm to compete at the nuclear pore for transport (nucleoplasmin is already in the nucleus). In our in vitro system, there are ~325–425 ng of unlabeled cytoplasmic nucleoplasmin per microliter of extract (1.7 egg equivalents/μl extract; 190–250 ng nucleoplasmin/egg [(Dingwall et al., 1982]). This represents an excess of 22–28-fold relative to the TRITC-nucleoplasmin present in the assay (~15 ng/μl of extract). Taking these factors into consideration, we calculate the maximum predicted accumulation in vitro to be 17–22-fold (i.e., $12 \times 40 \div 22$–28). The maximal observed accumulation with rat liver nuclei in vitro was 17.2-fold. Thus, this value is within the 17–22 range. (We have ignored the number of nuclear pores per square millimeter of nuclear envelope, but the pore density in oocyte nuclei is higher than that in rat liver nuclei [Maul, 1977].) Clearly, the amount of nucleoplasmin accumulated in the in vitro transport system closely approaches that seen for nucleoplasmin in vivo.

To characterize the molecular components of nuclear transport, we must ultimately replace the egg extract with a defined medium. Attempts to do this have not yet proved successful. When rat liver nuclei were added to a buffer similar to that in which the egg extract was made and supplemented with ATP, transport of TRITC-labeled nucleoplasmin was not observed. Our evidence indicates that membranes present in the egg extract are necessary for establishing transport competence in rat liver nuclei, but that these membranes can be reduced in amount later, since the extract plus nuclei can

be diluted 32-fold with membrane-free extract without affecting transport. The ease of the transport assay should prove valuable in testing a large number of defined media for support of in vitro transport. If, however, a cytoplasmic carrier protein is required to ferry nuclear proteins to the nucleus, any defined medium would have to be supplemented with that protein before transport would be observed in our assay.

Interestingly, Con A was found to have no noticeable effect on nucleoplasmin transport. Jiang and Schindler (1986) had previously found that 0.1 mg/ml Con A inhibits dextran influx into the nucleus, but only in the absence of ATP. The binding of Con A to the nuclear envelope has been observed in a number of previous studies. Ferritin-labeled Con A is known to bind to the cisternal faces of both the inner and outer nuclear membranes, but not to the nuclear or cytoplasmic faces of the membranes (Virtanen and Wartiovaara, 1976 and 1978; Feldherr et al., 1977; Seve et al., 1984; Schindler et al., 1985). It is possible, since we only see staining of damaged nuclei with FITC-Con A, that the envelopes of the affected nuclei in the dextran studies were not intact and the space between the nuclear membranes was accessible to Con A. The Con A then affected the diameter of the pore in an unknown manner, allowing less dextran into the nucleus. In our intact nuclei, Con A would have no effect since it is unable to reach its target, which is presumably the 180-kD Con A–binding pore protein identified by Gerace et al. (1982). (The sugar moiety of this protein lies within the space between the nuclear membranes.) It is also possible that Con A has no observable effect on nucleoplasmin transport in the presence of ATP. (Because of the absolute requirement for ATP, the effect of Con A on nucleoplasmin transport in the absence of ATP could not be tested.) Lastly, the differing response to Con A observed with nucleoplasmin transport and dextran influx through the nuclear pores may indicate that the mechanisms for these two processes differ significantly.

It should be possible using our in vitro transport assay to test models for the motive force of transport, such as those invoking a myosin Mg^{++}-ATPase (Fisher et al., 1985) or actin–myosin control of the pore diameter (Schindler and Jiang, 1986). Furthermore, with this in vitro system we should now be in a position to identify by direct biochemical means the pore proteins and perhaps cytoplasmic proteins involved in the transport process. With the isolated components of the transport machinery, it should then be possible to gain a molecular picture of the way in which transport occurs.

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