An *N*-ethylmaleimide-sensitive Cytosolic Factor Necessary for Nuclear Protein Import: Requirement in Signal-mediated Binding to the Nuclear Pore

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Abstract. We described previously an assay for authentic nuclear protein import in vitro. In this assay, exogenous nuclei are placed in an extract of *Xenopus* eggs; a rhodamine-labeled protein possessing a nuclear localization signal is added, and fluorescence microscopy is used to measure nuclear uptake. The requirement in this system for a cytosolic extract suggests that nuclear import is dependent on at least one cytosolic factor. We now confirm this hypothesis. Treatment of the cytosol with *N*-ethylmaleimide (NEM) abolishes nuclear protein import; readdition of a cytosolic fraction to the NEM-inactivated extract rescues transport. Thus, at least one NEM-sensitive factor required for transport is supplied by the cytosol. This activity, called nuclear import factor-1, or NIF-1, is ammonium-sulfate-precipitable, protease-sensitive, and heat-labile; it is therefore at least partly proteinaceous. NIF-1 stimulates, in a concentration-dependent manner, the rate at which individual nuclei accumulate protein. The effect of NIF-1 is enhanced by a second cytosolic NEM-sensitive factor, NIF-2. Earlier we identified two steps in the nuclear import reaction: (*a*) ATP-independent binding of a signal-sequence-bearing protein to the nuclear pore; and (*b*) ATP-dependent translocation of that protein through the pore. We now show that NEM inhibits signal-mediated binding, and that readdition of NIF-1 restores binding. Thus, NIF-1 is required for at least the binding step and does not require ATP for its activity. NIF-1 may act as a cytoplasmic signal receptor that escorts signal-bearing proteins to the pore, or may instead promote signal-mediated binding to the pore in another manner, as discussed.

THE migration of proteins into the cell nucleus is one example of the more general phenomenon of intracellular targeting: the delivery of macromolecules to their appropriate destinations in the cell. The mechanism of nuclear protein transport is of interest not only in its own right, but also because the regulated nuclear import of hormone receptors, protein kinases, and transcription factors may play an important role in relaying extracellular signals to the nucleus (e.g., Picard and Yamamoto, 1987; Cambier et al., 1987; Baeuerle and Baltimore, 1988*a*,*b*; Fujisawa-Sehara et al., 1988).

Nuclear protein import, as recent evidence shows, is actively mediated by pore complexes in the nuclear envelope (for reviews, see Dingwall and Laskey, 1986; Newport and Forbes, 1987; Newmeyer, 1989). Macromolecules <20–60 kD in molecular weight can diffuse through the nuclear pore and equilibrate between nucleus and cytoplasm. Larger macromolecules, however, are excluded from the nucleus unless they contain a nuclear targeting signal, in which case they not only pass through the pores (Feldherr et al., 1984) but accumulate rapidly inside the nucleus.

In recent years, deletion and point mutagenesis studies have identified the nuclear localization signals in several nuclear proteins. The best-studied signal is that of SV40 large T-antigen. A seven-amino-acid sequence (pro-lys-lys$_{128}$-lys-arg-lys-val) within this protein is both necessary and sufficient for its nuclear accumulation (Lanford and Butel, 1984; Kalderon et al., 1984*a*,*b*). Point mutations at lys$_{128}$ and, to a lesser extent, surrounding residues result in a defect in nuclear targeting (Lanford and Butel, 1984; Kalderon et al., 1984*b*; Smith et al., 1985; Lanford et al., 1988). Signal sequences or domains have been identified in several other nuclear proteins, including polyoma T-antigen (Richardson et al., 1986), nucleoplasmin (Bürglin and De Robertis, 1987; Dingwall et al., 1987, 1988) and others (Silver et al., 1984; Munro and Pelham, 1984; Davey et al., 1985; Moreland et al., 1985, 1987; Richter et al., 1985; Gritz et al., 1985; Wychowski et al., 1986; Lyon et al., 1987; Stone et al., 1987; Kleinschmidt and Seiter, 1988; Loewinger and McKeon, 1988; Dang and Lee, 1988). No clear consensus sequence for nuclear targeting has emerged. Although many of the known signals are rich in the basic amino acid residues lysine and arginine, it appears that overall charge is not the determining characteristic, but rather how the charges are posi-

tioned spatially (Lanford et al., 1988). The SV40 T-antigen signal sequence seems to act as an autonomous entity, since its function is largely (although not entirely) insensitive to location in the primary sequence of the protein (Roberts et al., 1987). Moreover, this peptide is recognized as a signal even when chemically cross-linked to a protein (Goldfarb et al., 1986; Lanford et al., 1986). Chelsky et al. (1989) found that signals from other proteins also function in this manner and suggested a consensus sequence (lys; arg/lys; X; arg/lys) for peptide-mediated nuclear targeting. On the other hand, Rihs et al. (1989) found that the simple SV40 T-antigen signal is not sufficient for rapid nuclear uptake of microinjected fusion proteins, which contain only one signal per protein; flanking sequences are also required. This suggests the possibility that the kinetics of nuclear protein transport can be subtly regulated.

Such studies have illuminated the properties of nuclear localization signals. Less is known, however, about the detailed mechanism of nuclear protein import. To help understand the transport mechanism, we developed an in vitro nuclear import system using extracts from *Xenopus* eggs (Newmeyer et al., 1986*a*,*b*). Studies with this system have shown that nuclear import requires ATP and an intact nuclear envelope (Newmeyer et al., 1986*a*,*b*; Newmeyer and Forbes, 1988). Nuclear protein accumulation is therefore not a process based on simple diffusion through the pore and intranuclear binding, but instead is mediated actively by the nuclear pore.

The very existence of nuclear targeting signals implies that a signal-recognition molecule is involved in nuclear protein import. Experimental evidence supports this idea. Goldfarb et al. (1986) found that the nuclear import in vivo of a protein conjugated with the SV40 T-antigen signal peptide displayed saturable kinetics. Furthermore, the initial rate of accumulation was lowered by coinjection of excess free wild-type signal peptide and, to a lesser extent, by a mutant peptide in which the equivalent of lys_{128} was changed to thr. This argues that nuclear import is a carrier-mediated process. Using our in vitro system, we found direct evidence for a signal-sequence binding event in nuclear import (Newmeyer and Forbes, 1988). With electron microscopy, we examined the transport through individual nuclear pores of colloidal gold particles complexed with HSA-signal peptide conjugates (a method developed by Feldherr et al., 1984). We found that in the absence of ATP, protein-gold complexes were not translocated through the pores. Instead, the particles were trapped at an intermediate step, bound to the cytoplasmic face of the nuclear pore. Similarly, we found that the lectin WGA, previously shown to block nuclear protein accumulation (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988), inhibited translocation through the pore. As a result, gold particles were arrested at the cytoplasmic face of the pore. These results show that the nuclear import mechanism is composed of at least two separable steps. The first step, signal-mediated binding of a nuclear protein to the cytoplasmic face of the nuclear pore, does not require ATP and is not blocked by the transport inhibitor, WGA. In contrast, the second step, translocation through the central channel of the pore, is ATP-dependent and inhibited by the binding of WGA to nuclear pore glycoproteins. Richardson et al. (1988) obtained complementary results in vivo: when the nuclear protein nucleoplasmin was microinjected into cultured cells, it appeared first to bind to the nuclear envelope and then to be translocated through the pores. At low temperatures or under conditions of metabolic inhibition (incubation with sodium fluoride and 2-deoxyglucose), translocation, but not pore binding, was inhibited.

Signal-dependent binding to the nuclear pore is most likely mediated by a signal-sequence "receptor." Such a receptor might be permanently resident at the pore. Or, it might be a cytoplasmic "carrier" that escorts the signal-containing protein to the pore, in analogy to the signal-recognition particle involved in protein transport into the endoplasmic reticulum (e.g., Walter and Blobel, 1980; Walter et al., 1984). Recent studies have identified polypeptides that may function as receptors for nuclear localization signals (Yoneda et al., 1988; Adam et al., 1989; Yamasaki et al., 1989). The proteins studied by Yoneda et al. (1988) were detected exclusively in the nucleus. However, the other two groups found signal-binding proteins present in cytosolic fractions as well. While these results are intriguing, conclusive evidence that any of these candidate receptor proteins indeed function as signal receptors in vivo is still awaited.

Knowledge of the cellular location of the signal-sequence receptor, or of any factor required for nuclear protein import, would be an important clue to understanding the transport mechanism. In this report, we pose the question of whether nuclear protein import requires the participation of cytosolic factors. The *Xenopus* in vitro system used for our studies is based on an egg extract, a complex mixture of organelles and macromolecules. This extract is known to contain all the activities needed for nuclear membrane assembly and growth (Lohka and Masui, 1983, 1984; Newport and Forbes, 1985; Newmeyer et al., 1986*a*,*b*; Newport, 1987; Wilson and Newport, 1988), DNA replication (Blow and Laskey, 1986, 1988; Newport, 1987; Blow and Watson, 1987; Hutchison et al., 1988), and other functions. One would predict that a much smaller set of activities is sufficient for nuclear import. The simplest hypothesis might be that isolated nuclei contain all the components necessary for protein uptake. A direct test of this hypothesis would be to ask whether isolated nuclei placed in a defined buffer containing ATP or other cofactors can accumulate protein in a signal-dependent manner. However, we found that this direct approach was subject to artifacts of nonspecific binding; it could not be demonstrated that the association of proteins with isolated nuclei reflected active transport through nuclear pores. Instead, we made use of a strategy previously employed to identify a cytosolic factor, NSF, involved in transport within the Golgi (Glick and Rothman, 1987; Block et al., 1988). With this approach, based on the treatment of cytosol with the protein modifier, *N*-ethylmaleimide (NEM)[1], we found that at least two cytosolic factors, named NIF-1 and NIF-2 (for NIF, nuclear import factor), participate in nuclear import. At least one of them, NIF-1, is required for the signal-mediated binding of a nuclear protein to the nuclear pore.

Materials and Methods

Preparation of Extracts and Nuclei

Preparations of *Xenopus* egg extracts and rat liver nuclei were as described previously (Newmeyer et al., 1986*b*; Newport, 1987; Newport and Spann,

1. *Abbreviations used in this paper*: ELB, egg lysis buffer, HSA, human serum albumin; NEM, *N*-ethylmaleimide; NIF, nuclear import factor.

1987) except that eggs were not activated before lysis. Leupeptin (50 μg/ml) and aprotinin (50 μg/ml) were added to the extracts, except in experiments testing trypsin-sensitivity. Cytosol and membrane fractions were prepared by ultracentrifugation in (TL-100 centrifuge; Beckman Instruments, Inc., Palo Alto, CA), with a TLS-55 rotor, at 45,000 RPM for 1.5 h at 4°C. The supernatant was carefully withdrawn from the side with a 22-ga needle. After removing as much of the supernatant as possible, the membrane layer overlying the amber-colored pellet of glycogen and ribosomes was removed with a wide-bore 200-μl pipet tip. The supernatant (cytosol) was quick-frozen in liquid nitrogen and stored at −80°C; the membrane fraction was mixed with 1/10 vol DMSO and 60-μl aliquots were placed directly at −80°C.

Standard Nuclear Import Assay Using Unfractionated Extracts

The nuclear import assay was essentially as described by Newmeyer et al. (1986*b*). Rat liver nuclei (5×10^5) were placed in 10 μl of egg extract containing an ATP-regenerating system (1 mM ATP, 10 mM phosphocreatine, and 100 U/ml creatine kinase). After a 15-min incubation, 0.5 μl (160 ng) of the transport substrate was added. The transport substrate, TRITC-HSA-wt (Newmeyer and Forbes, 1988) was a conjugate of human serum albumin (HSA) and a synthetic peptide (cys-thr-pro-pro-lys-lys-lys-arg-lys-val-COOH) containing the nuclear localization signal of SV40 large T-antigen. Each molecule of HSA carried ~20 peptide molecules, on average, as determined by SDS gel electrophoresis. At various times (30–180 min) after the addition of TRITC-HSA-wt, a 3.5-μl aliquot of the assay mixture was withdrawn, mixed with 0.5 μl of a solution of 37% formaldehyde and 10 μg/ml Hoechst 33258 (a DNA fluorochrome), and examined by fluorescence microscopy.

Reconstitution of Nuclear Import Extracts from Cytosol and Membrane Fractions

A 60-μl aliquot of membranes was thawed and washed in 3.6 ml of egg lysis buffer (ELB): 20 mM K-Hepes, pH 7.4, 50 mM KCl, 250 mM sucrose, 1 mM DTT, and 5 μg/ml cytochalasin B. Membranes were pelleted for 15 min in a microfuge (Beckman Instruments, Inc.) at 4°C. Just before use they were resuspended in an equal volume (~20 μl) of ELB. To reconstitute the nuclear import system from membrane and cytosolic components, the washed membranes were mixed with 80 μl of cytosol and an ATP-regenerating system. Rat liver nuclei were added and incubated for 15 min before addition of the transport substrate and subsequent measurement of transport.

Assay for NEM-sensitive Factors

The assay for NEM-sensitive cytosolic factors was performed as follows: cytosol was treated with 5 mM NEM for 15 min at 23°C before addition of DTT (10 mM) to inactivate remaining unreacted NEM. The NEM-treated cytosol was then combined with an aliquot of washed, non-NEM-treated membranes, rat liver nuclei ($5 \times 10^4/\mu l$), and an ATP-regenerating system (Newmeyer et al., 1986*b*). The mixture was divided into 9-μl aliquots. To each aliquot was added 3 μl of either (*a*) egg lysis buffer; (*b*) untreated cytosol; (*c*) NEM-treated cytosol; or (*d*) a cytosolic fraction to be tested for transport-stimulating activity. Finally, 0.5 μl (160 ng) of the TRITC-HSA-wt transport substrate was added to each tube, using an ultra-micro pipettor (Eppendorf Instruments made by Brinkmann Instruments, Inc., Westbury, NY). After an incubation of 30 min to 2 h, a 3.5-μl aliquot of each sample was withdrawn, mixed on a microscope slide with 0.5 μl of the Hoechst 33258/formaldehyde mixture, and examined in the fluorescence microscope. The extent of nuclear accumulation of the fluorescent protein was measured by densitometric scanning of photographic negatives, as previously described (Newmeyer et al., 1986*b*), taking into account the slight dilution with fixative. ATP depletion experiments were performed by pretreating the extracts with the enzyme apyrase, as described by Newmeyer et al. (1986*a*).

It should be noted that both the cytosol and membrane fractions are required for nuclear import, even when nuclei are added exogenously (our unpublished data). We believe that the membrane fraction is required primarily for nuclear membrane healing and stability (Lohka and Masui, 1984; Wilson and Newport, 1988) rather than for the transport mechanism per se. For reasons yet unknown, when rat liver nuclei are placed in cytosol alone, the nuclei lyse, spilling out much of their chromatin. Nuclear proteins cannot accumulate in such damaged nuclei (Newmeyer et al., 1986*b*). Apparently the cytosol contains a nuclear lytic activity; the presence of membranes, however, prevents lysis. We found that NEM treatment of the membrane fraction (5 mM NEM, 15 min, 23°C, followed by addition of DTT to 10 mM) not only inhibited nuclear membrane assembly and growth, but gave a result similar to omitting the membrane fraction entirely; the nuclei lysed and no nuclear import activity could be observed. Thus the membrane pellet contains an NEM-sensitive factor (or factors) required for nuclear membrane stability and growth. This factor may be similar or identical to the membrane fusion factor, NSF, required for vesicular transport within the Golgi apparatus (Glick and Rothman, 1987; Block et al., 1988; Malhotra et al., 1988). We think that the NEM-sensitive factor in our membrane fraction is only indirectly relevant to the nuclear import process, inasmuch as it contributes to nuclear stability, a prerequisite for import. Therefore, to test for cytosolic NEM-sensitive transport factors, untreated membranes were routinely included in all assay mixtures (except in the experiment shown in Fig. 1) to prevent nuclear lysis. With regard to Fig. 1, we note that the nuclear lytic activity is inactivated by NEM, since nuclei placed in NEM-treated unfractionated egg extracts, which contain both membranes and cytosol, did not lyse.

Ammonium Sulfate Precipitation of NIF-1 from Egg Cytosol

To determine whether NIF-1 was ammonium-sulfate-precipitable, the cytosol was diluted 1:1 with ELB, and a solution of 100% saturated ammonium sulfate was added to a final percentage of saturation ranging from 20 to 80%. After 30 min at 0°C, samples were pelleted in a centrifuge (Eppendorf made by Brinkmann Instruments, Inc., Westbury, NY) for 2 min. The pellets were washed in a solution of the same percentage saturated ammonium sulfate and recentrifuged. Finally, the material was resuspended in one-eighth the original volume of ELB containing 50 μg/ml aprotinin and 50 μg/ml leupeptin, dialyzed in a microdialyzer apparatus (Pierce Chemical Co., Rockford, IL) for 3–4 h against the same buffer, and frozen in aliquots at −80°C. It was found that NIF-1 activity was salted out when ammonium sulfate was added to between 30 and 50% saturation. At the higher salt concentrations, however, proteins precipitated that resulted in increased formation of fibrillar networks around the nuclei in the reconstituted extracts. Formation of these networks had no detrimental effect on nuclear protein import, but caused difficulties in handling the extracts. Precipitation with 40% saturated ammonium sulfate reduced this problem while permitting recovery of the majority of the NIF-1 activity, and was therefore used routinely.

Video Image Analysis

For the experiment shown in Fig. 5, the nuclear accumulation of the transport substrate TRITC-HSA-wt was quantitated using a video image analysis system. A Dage MTI CCD-72 video camera was mounted on a Nikon Optiphot fluorescence microscope with a Nikon 60× plan-apochromat objective. Images were captured with an Imaging Technologies PC Vision Plus 512 frame-grabber board installed in an IBM AT-compatible computer, and displayed on a black-and-white video monitor. Optimas software (Bioscan, Inc., Edmonds, WA) was used to collect luminance data. Brightness and contrast were calibrated using the test pattern generated by the video camera. All controls on the video camera were set to manual. The black level was set so that the background light level in the optical system (i.e., in a specimen containing no fluorescent protein) was slightly above zero; this background value was subtracted from all measurements. The video camera gain was set at 5.0, so that the brightest nuclei did not exceed the camera's range. Nuclei were selected at random; damaged nuclei (which do not accumulate protein; Newmeyer et al., 1986*b*) were excluded from the measurements. An area inside each nucleus was marked with the mouse, and the average luminance (fluorescence intensity) in that region was calculated. For each sample, the luminances of 15–25 nuclei were averaged and the standard error calculated. Luminances are expressed in arbitrary units in a range of 0–255, the values encodeable by the eight-bit frame grabber.

Results

NEM Treatment of a Cell-free Extract Abolishes Nuclear Import Activity

Cytosolic factors are required for the import of proteins into the endoplasmic reticulum and into mitochondria (e.g., Walter and Blobel, 1980; Waters et al., 1986; Deshaies et al., 1988; Murakami et al., 1988; Siegel and Water, 1988; Bankaitis et al., 1989). To ask whether cytoplasmic factors are

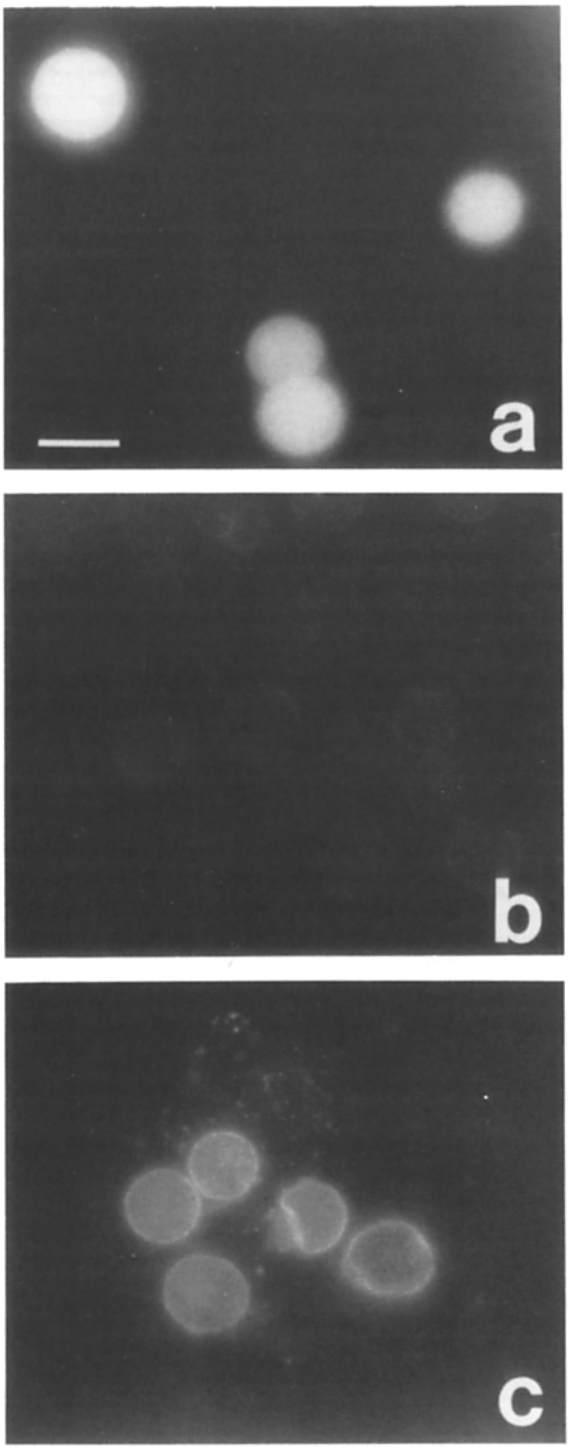

Figure 1. NEM treatment of the egg extract inhibits nuclear protein import, specifically at the step of signal-dependent nuclear pore binding. (*a*) DTT (10 mM) was added to the extract just before the addition of NEM (5 mM). An ATP-regenerating system, rat liver nuclei, and the transport substrate, TRITC-HSA-wt, were then added. After a 30-min incubation, a 3.5-μl aliquot was mixed with 0.5 μl of a solution of Hoechst 33258 (100 μg/ml) in 37% formaldehyde and examined by fluorescence microscopy. The TRITC fluorescence is shown. *b* is the same as *a*, except that the extract was incubated in the presence of NEM (5 mM) for 15 min at 22°C before inactivation of NEM by the addition of DTT (10 mM); (*c*) the extract was treated with 10 mM DTT and depleted of ATP by incubation in the presence of 2 U of apyrase per ml for 30 min at 22°C before the addition of rat liver nuclei and TRITC-HSA-wt. Note that in *b* both nuclear accumulation and nuclear envelope binding of TRITC-HSA-wt are inhibited. Bar, 10 μm.

also involved in nuclear protein import, we made use of a system with which nuclear import can be observed and manipulated in vitro (Newmeyer et al., 1986*a*,*b*; Finlay et al., 1987; Newmeyer and Forbes, 1988). This system employs an extract of *Xenopus* eggs, nuclei added from an exogenous source (e.g., rat liver), and a fluorescent protein transport substrate capable of being imported by cell nuclei. The transport substrate used was HSA, conjugated with multiple copies of a synthetic peptide corresponding to the nuclear targeting signal of the wild-type SV40 large T-antigen, and labeled with the fluor, tetramethylrhodamine. The conjugate (abbreviated TRITC-HSA-wt) is transported efficiently by rat liver nuclei in the in vitro system (see Fig. 1 *a*); a conjugate of HSA with a mutant signal peptide, in which the lysine corresponding to residue 128 in T-antigen is changed to threonine, is excluded by nuclei (Newmeyer and Forbes, 1988).

Nuclear import in this in vitro system has been shown to be authentic by several criteria (Newmeyer et al., 1986*b*; Newmeyer and Forbes, 1988). First, transport is specific in that it depends on the presence of a functional nuclear localization signal in the protein substrate. Second, protein accumulation occurs only in intact nuclei; thus the system displays true nuclear envelope-mediated transport, rather than the diffusion of proteins through tears in the nuclear envelope and the binding of those proteins to immobile elements inside the nuclei. Third, the nuclear import observed is ATP- and temperature-dependent. Finally, transport is mediated by the nuclear pores: electron microscopy has shown that colloidal gold particles, complexed with transport substrates and added to the in vitro transport assay, can be found being translocated through the pore channels (Newmeyer and Forbes, 1988).

One would like ultimately to reconstitute the nuclear transport system from purified components. It has been unclear whether factors other than those found in the nuclear pore are required. To search for factors involved in the mechanism of nuclear import, we tested the effect of the compound *N*-ethylmaleimide (NEM), a sulfhydryl alkylating agent, on the in vitro nuclear import system. If NEM were to inactivate one or more factors required for nuclear import, the resulting deficient system could then be supplemented by various biochemical fractions to test for the rescue of transport activity. Such a biochemical complementation approach has aided the study of protein transport within the Golgi apparatus (Glick and Rothman, 1987; Malhotra et al., 1988; Block et al., 1988), into mitochondria (Murakami et al., 1988), and into the endoplasmic reticulum (Siegel and Walter, 1988).

To test the effect of NEM in the transport assay, an aliquot of unfractionated egg extract (containing both membranes and cytosol) was incubated for 15 min at 23° in the presence of NEM (5 mM). DTT (10 mM) was then added to inactivate excess unreacted NEM. Finally, rat liver nuclei and TRITC-HSA-wt were added and transport was assayed by fluorescence microscopy 30 min later. We found that a substantial proportion of the nuclei were intact under these conditions, as judged by their ability to exclude fluoresceinated IgG. However, nuclear import activity in these nuclei was reduced

to a barely detectable level (Fig. 1 *b*). In a control experiment, in which the order of addition of NEM and DTT was reversed (and hence the NEM was inactivated from the start), the TRITC-HSA-wt substrate was accumulated normally by rat liver nuclei (Fig. 1 *a*). We conclude that an NEM-sensitive factor (or set of factors) required for nuclear import resides in the extract, and that isolated nuclei added to the extract do not contain sufficient amounts of this factor for transport to be detectable in our assay.

It was shown previously that the nuclear import reaction is composed of two steps: (*a*) ATP-independent binding of signal-bearing proteins to the pore; and (*b*) ATP-dependent translocation of the proteins through the pore (Newmeyer and Forbes, 1988; Richardson et al., 1988). When ATP is removed from the extract by addition of the enzyme apyrase, TRITC-HSA-wt binds to, but cannot be translocated through, the nuclear pores. This nuclear pore binding manifests itself as a ring of fluorescence at the nuclear periphery (Fig. 1 *c*). When the plane of focus is at the surface of the nuclei, a punctate staining pattern can often be seen, as in Fig. 4 *c*. Electron microscopy has demonstrated that this punctate staining is due to association of TRITC-HSA-wt with the nuclear pores (Newmeyer and Forbes, 1988). From Fig. 1 *b* we determined that NEM treatment of the extract inhibits not only the nuclear accumulation of the transport substrate, but also its binding to the nuclear pores. The very small amount of fluorescence observed at the nuclear periphery in Fig. 1 *b* may be because of either nonspecific binding or to a small amount of signal-dependent binding activity contributed by the nuclei themselves, perhaps because of contamination of the nuclei with rat liver cytosol. (Because binding is a prerequisite for translocation, this experiment does not address the question of whether NEM may also inhibit the translocation step.) We conclude that NEM inactivates a factor in the extract that is essential for signal-mediated binding to the nuclear pore.

A Cytosolic Factor, NIF-1, Restores Nuclear Import Activity to the NEM-treated Extract

The results above suggest that there is at least one NEM-sensitive factor in the extract that is required for nuclear import, and in particular, for binding of the transport substrate to the nuclear pore. To begin characterizing such factors, we separated the extract by high-speed centrifugation into soluble (cytosol) and membrane fractions (see Materials and Methods). Both fractions are required for nuclear import activity. The NEM-sensitivity of the unfractionated extract might be because of a requirement for NEM-sensitive factors in either the cytosol or membrane fractions, or both. Initial experiments showed that the membrane fraction contains at least one NEM-sensitive factor required for nuclear membrane stability (see Materials and Methods). However, this factor is most likely only indirectly relevant to the nuclear import process, inasmuch as intactness of the nuclear membrane is a prerequisite for protein accumulation. All experiments described below were performed in the presence of untreated membranes.

To determine whether there is an NEM-sensitive factor required for transport in the soluble fraction of the extract, the cytosol was treated with NEM (5 mM) for 15 min and then the NEM inactivated by the addition of DTT (10 mM). The NEM-treated cytosol was recombined with untreated membranes. Intact nuclei placed in this reconstituted extract were stable and continued to exclude large nonnuclear proteins such as FITC-IgG for at least 2 h (not shown). Unlike control extracts, in which rat liver nuclei undergo considerable membrane growth (Newmeyer et al., 1986*b*), nuclear membranes were observed to grow only slightly when nuclei were placed in the mixture of untreated membranes and NEM-treated cytosol. In the reconstituted extract containing NEM-treated cytosol and untreated membranes, we found that nuclear import occurred at only about one-tenth of the normal rate (Fig. 2 *a*). The residual transport observed with the NEM-treated cytosol may be because of a slight contamination of the washed membrane fraction with untreated cytosol. We conclude from the observed transport inhibition that a cytosolic component sensitive to NEM is required for efficient nuclear import.

We asked next whether nuclear import activity could be rescued in this deficient system by supplementation with crude cytosol. The addition of one-third volume of untreated cytosol to this mixture (consisting of two parts NEM-treated cytosol, one part untreated membranes, and nuclei) caused many of the nuclei to lyse (see Materials and Methods); however, the remaining intact nuclei displayed high nuclear import activity (not shown). We found, as shown below, that this restoration of nuclear transport by cytosol provided a biochemical complementation assay for the NEM-sensitive cytosolic factor required for nuclear import once the problem of nuclear lysis was circumvented.

As a first step in purifying the NEM-sensitive nuclear import factor (henceforth referred to as NIF-1), we fractionated the cytosolic extract by salt precipitation. Ammonium sulfate was added in various amounts to the crude extract, and after 30 min, the precipitate was collected by centrifugation, resuspended in egg lysis buffer, and dialyzed against the same buffer. The resulting preparation was tested for the ability to restore nuclear transport activity to the assay system. Specifically, two parts NEM-treated cytosol were combined with one part membranes, and an aliquot of rat liver nuclei. To this mixture one-third volume of the ammonium sulfate pellet fraction or buffer was added; finally the substrate TRITC-HSA-wt was added and transport assayed. We found that NIF-1 activity was recovered after precipitation with 40% saturated ammonium sulfate. Addition of the dialyzed ammonium-sulfate precipitate containing NIF-1 (henceforth abbreviated "AP-NIF-1") to NEM-treated cytosol restored nuclear import activity to high levels, without causing nuclear lysis (Fig. 2, *c* and *d*). Evidently the nuclear lytic activity found in the crude extract is soluble in, or inactivated by, 40% saturated ammonium sulfate. NIF-1 was destroyed by trypsin or heat treatment (80°, 5 min) (not shown); therefore, we conclude that NIF-1 is at least partly proteinaceous. In preliminary investigations, when cytosol or AP-NIF-1 was chromatographed on Sephacryl S-300, transport-stimulating activity was present in the excluded peak (corresponding to an apparent native molecular mass of over 500 kD) and in a second peak near 50 kD (data not shown). This suggests that NIF-1 activity can occur in a macromolecular complex.

In a similar experiment, we found that AP-NIF-1 was required for transport of the *Xenopus* oocyte nuclear protein, nucleoplasmin (not shown). Thus, NIF-1 plays a role in both the import of the synthetic transport substrate HSA-wt and

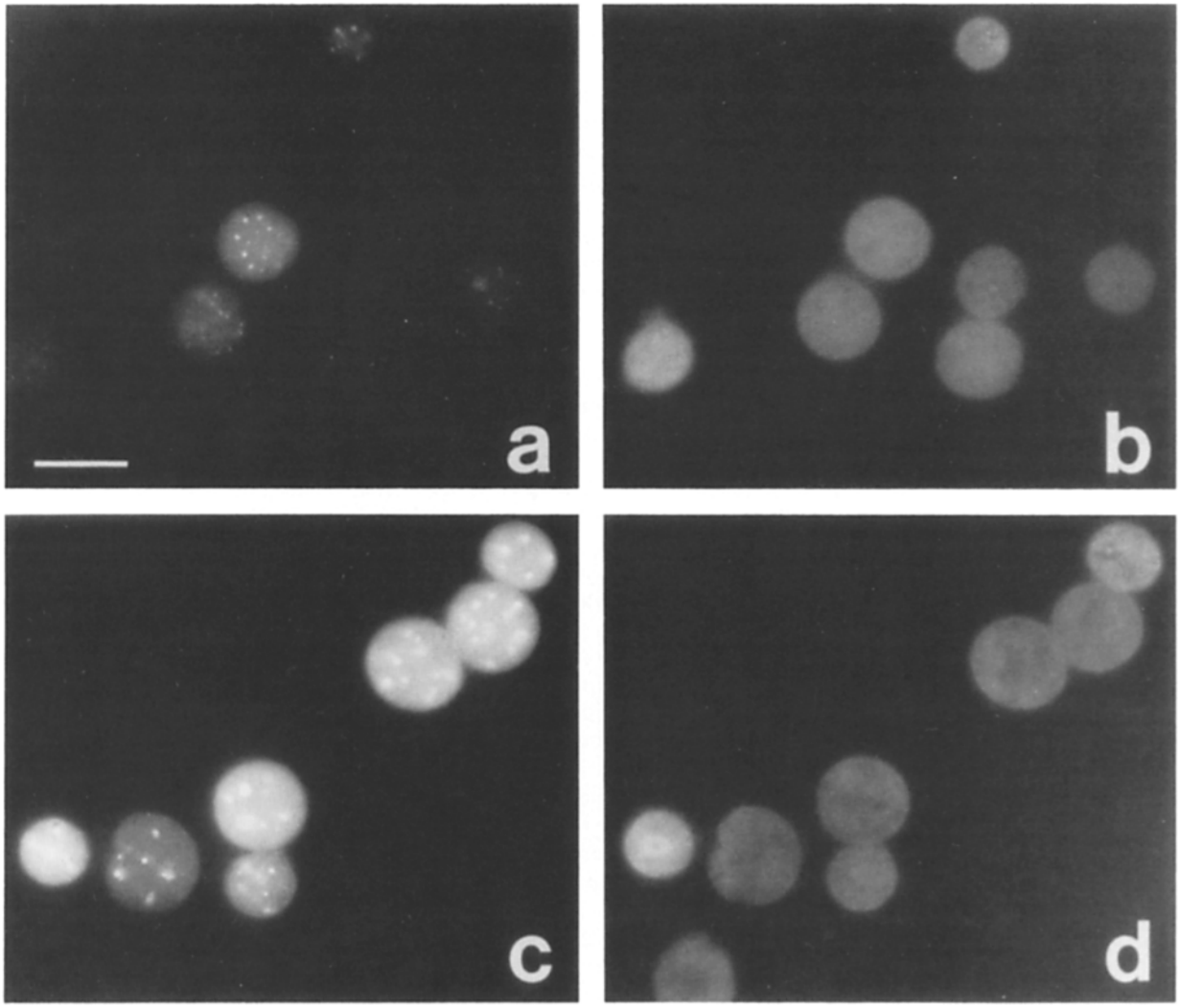

Figure 2. The addition of a cytosolic fraction (AP-NIF-1) restores transport activity to an assay mixture containing NEM-treated cytosol and untreated membrane vesicles. (*a* and *b*) Buffer (3 μl), an ATP-regenerating system, rat liver nuclei, and TRITC-HSA-wt were added to a 9-μl aliquot of a mixture of NEM-treated cytosol and untreated membranes. After 1 h, an aliquot was examined by fluorescence microscopy as in Fig. 1. (*c* and *d*) same as (*a* and *b*) except that instead of buffer, a 3-μl aliquot of AP-NIF-1 was added. In this experiment, the ammonium sulfate pellet was dissolved in a volume of buffer equal to one-fourth the original volume of the extract from which it was derived. (*a* and *c*) TRITC fluorescence; (*b* and *d*) Hoechst 33258 fluorescence (DNA). Note that in *c*, transport has been restored by the addition of AP-NIF-1. Bar, 10 μm.

in the import of a native nuclear protein containing a signal sequence different from that of the SV40 T-antigen.

NIF-1 Is Required Directly for Transport Function rather than for Nuclear Membrane Stability

Since nuclear integrity is a prerequisite for nuclear protein accumulation, it was formally possible that NIF-1 stimulates transport indirectly by increasing the stability of the nuclear envelope. If so, one would predict that increasing amounts of NIF-1 added to the extract would increase the number of intact nuclei. However, we found that not to be the case. NIF-1 caused no significant change in the percentage of intact nuclei as measured by the exclusion of fluorescently labeled IgG. In one typical experiment, intact nuclei represented 56% of the population before, and 61% after, addition of AP-NIF-1.

Instead of increasing the number of intact, transport-competent nuclei, NIF-1 was found to stimulate the rate of protein accumulation. To measure nuclear accumulation of the TRITC-HSA-wt transport substrate in individual nuclei, the nuclei were photographed, a densitometer was used to scan the negatives, and the ratio of intranuclear to extranuclear fluorescence intensities was calculated (Newmeyer et al., 1986*b*). As seen in Fig. 3, we found that the accumulation of TRITC-HSA-wt in intact nuclei was dependent on the amount of AP-NIF-1 added. The response was linear at lower concentrations of AP-NIF-1, but reached a plateau when AP-NIF-1 was added in amounts roughly corresponding to those present in the original extract (assuming 100% recovery of NIF-1 activity in the ammonium-sulfate-precipitable fraction). The exact degree to which AP-NIF-1 stimulated transport varied between preparations. However, an increase of

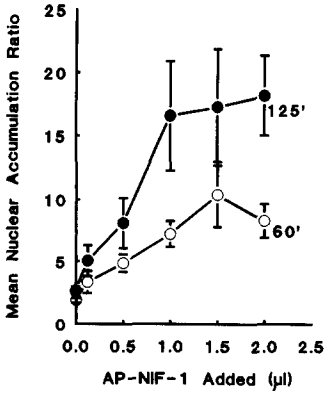

Figure 3. NIF-1 stimulates the rate of nuclear protein import in individual nuclei. The transport assay was essentially the same as in Fig. 2. Aliquots (6 μl) of a mixture of NEM-treated cytosol and untreated membrane vesicles were supplemented with the indicated amounts of AP-NIF-1 (protein concentration was 12 mg/ml), and the total volume was brought to 8 μl with egg lysis buffer. Then the transport substrate TRITC-HSA-wt was added. After incubations of 60 min (*open circles*) and 125 min (*solid circles*), 3.5-μl aliquots were withdrawn and examined by fluorescence microscopy. Nuclei accumulating the transport substrate were chosen at random and photographed. The accumulation ratios (internal versus external fluorescence) of TRITC-HSA-wt in individual nuclei were measured by densitometric scanning of photographic negatives, as described in the text. The values plotted are averages over at least eight measurements. The error bars represent SEM. Note that AP-NIF-1 stimulated nuclear import approximately sixfold in this experiment, a typical result.

approximately sixfold was commonly seen; occasional preparations displayed smaller (threefold) or greater (tenfold) stimulation.

We believe that the stimulation of nuclear import by NIF-1 cannot be because of assembly of new nuclear pores. If it were, prolonged preincubation in the presence of NIF-1 should make nuclear import more efficient upon later addition of a transport substrate. However, neither the extent of transport in a 30-min interval nor the number of intact nuclei was increased when a 2 h preincubation in the presence of NIF-1 was performed before the addition of TRITC-HSA-wt, as compared with simultaneous incubation with NIF-1 (not shown). We conclude that NIF-1 influences neither nuclear stability nor pore assembly, but rather is required directly for the nuclear import mechanism.

NIF-1 Participates in Signal-mediated Binding to the Pore

What role does NIF-1 play in the nuclear import mechanism? Since NEM treatment of the cytosol inhibits binding of the signal peptide to the pore (Fig. 1 *b*), one might predict that addition of NIF-1 to an NEM-treated extract would restore binding. We tested this prediction, taking advantage of our earlier observation that the pore binding step can be distinguished experimentally from the translocation step by removal of ATP (Newmeyer and Forbes, 1988). The assay mixture (containing NEM-treated cytosol, untreated membranes, and untreated rat liver nuclei), with or without added AP-NIF-1, was depleted of ATP with the enzyme apyrase (Newmeyer et al., 1986*a*). TRITC-HSA-wt was added and an aliquot of the mixture was observed in the fluorescence microscope 30 min later. We found, as before, that only dim fluorescence was visible at the nuclear rim in the NEM-treated extract (Fig. 4 *a*). However, addition of AP-NIF-1 resulted in a marked increase in the amount of TRITC-HSA-

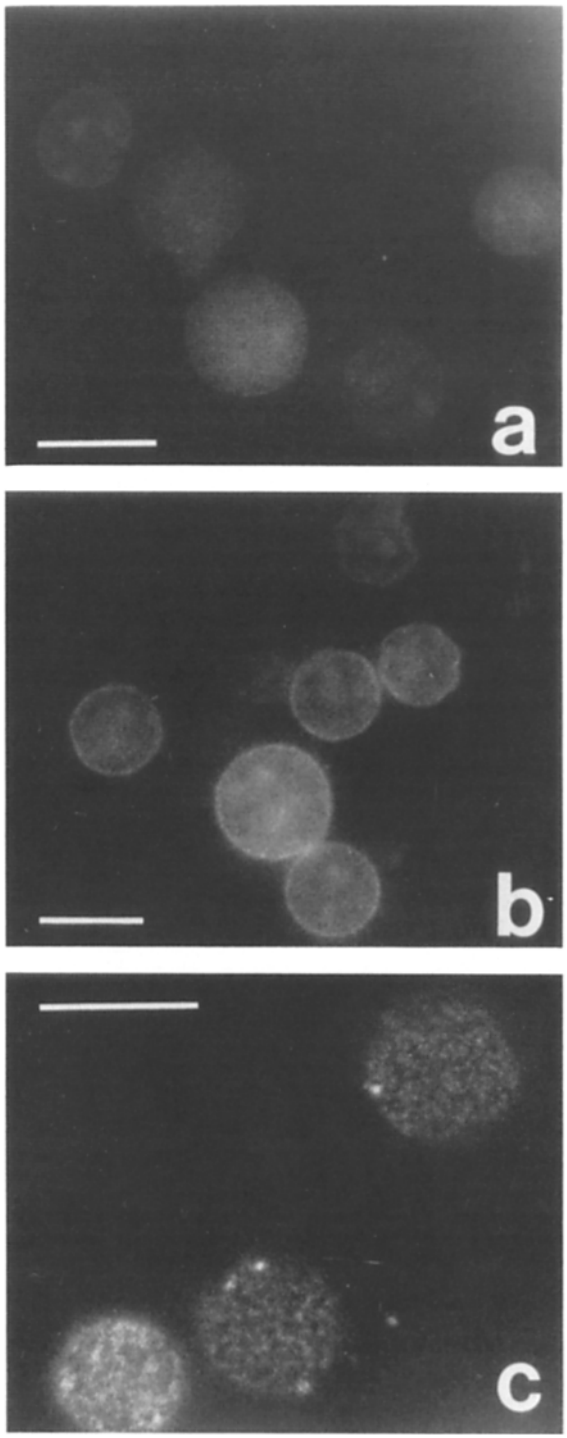

Figure 4. NIF-1 increases binding of TRITC-HSA-wt to the nuclear pore. Nuclei were incubated for 30 min in a mixture of NEM-treated cytosol and untreated membranes, supplemented either by buffer (*a*) or by AP-NIF-1 (*b* and *c*). ATP was removed from the samples by a further 30-min incubation in the presence of apyrase (2 U/ml). The transport substrate, TRITC-HSA-wt, was then added, and, after a final incubation of 30 min, aliquots were examined by fluorescence microscopy. TRITC fluorescence is shown. Note that addition of AP-NIF-1 increases the nuclear envelope binding of TRITC-HSA-wt (*b*); this binding displays a punctate pattern when the plane of focus is at the nuclear surface (*c*), indicating association with the nuclear pores (Newmeyer and Forbes, 1988). Bars, 10 μm.

wt fluorescence located at the nuclear rim (Fig. 4 *b*). When the plane of focus was at the surface of the nuclei, a punctate pattern was observed (Fig. 4 *c*), characteristic of nuclear pore binding (Newmeyer and Forbes, 1988). Similar results were obtained after shorter incubations (5 min) with TRITC-HSA-wt. We concluded above that NEM inactivates the binding step; we now can conclude that NIF-1 restores the binding of TRITC-HSA-wt to the nuclear pore. NIF-1 therefore is required for the binding step of the transport reaction. Because these experiments were performed in the absence of ATP, it can also be concluded that NIF-1 does not need ATP for its action.

A Second NEM-sensitive Factor, NIF-2, Acts Synergistically with NIF-1

It was observed above that NIF-1 stimulates nuclear import in a concentration-dependent manner (Fig. 3). The response curve is biphasic: at low levels of NIF-1, the curve is linear, while at higher inputs of NIF-1, the level of transport reaches a plateau, suggesting that NIF-1 is no longer the limiting factor in the extract. To determine whether other factors in the extract cooperate with NIF-1 to promote nuclear import, we did the following experiment. The assay mixture (NEM-treated cytosol, untreated membranes, an ATP-regenerating system, and rat liver nuclei) was supplemented with either buffer or a small amount of untreated cytosol (the final concentration representing a 1:24 dilution of the standard cytosol preparation). Previous experiments had shown that the addition of this very small amount of untreated cytosol alone did not cause lysis or stimulate nuclear import detectably in our assay. Various amounts of AP-NIF-1 were then added to the above mixture, followed by the transport substrate, TRITC-HSA-wt. The mixture was incubated at 22°C, and aliquots were removed at various times and examined in the fluorescence microscope. The extent of nuclear import of TRITC-HSA-wt was quantitated using a video image analysis system (see Materials and Methods).

In Fig. 5 *a*, it may be seen that the addition of untreated cytosol at a 1:24 dilution did not by itself produce any detectable stimulation of nuclear protein import in a 150-min incubation. However, it did enhance significantly the effect of added AP-NIF-1. When diluted cytosol was added to the assay mixture, the response to NIF-1 was greater by ~75% than in the control (buffer added instead of cytosol). Although addition of dilute cytosol reproducibly yielded a synergistic effect on the action of AP-NIF-1, the degree of enhancement was somewhat variable between preparations of cytosol. Fig. 5 *b* shows a separate experiment done with different preparations of egg cytosol, membranes, and AP-NIF-1; nuclei were incubated for 60 min after addition of the transport substrate. Here, the addition of a 1:24 dilution of untreated cytosol stimulated transport somewhat even when AP-NIF-1 was not added, suggesting that this preparation of cytosol contained higher levels of transport factors than the cytosol preparation used for Fig. 5 *a*. Nevertheless, levels of transport continued to rise as increasing amounts of AP-NIF-1 were added. In this experiment, the addition of untreated cytosol resulted in an enhancement of approximately two-fold in the effect of AP-NIF-1. The combined effect of dilute cytosol and AP-NIF-1 was to stimulate transport more than 10-fold over the basal level. We conclude from these experiments that at least one other cytosolic NEM-sensitive factor, which we call NIF-2, can stimulate the nuclear import process; moreover, NIF-1 and NIF-2 act synergistically.

Since the synergistic effect of NIF-2 is seen even when adding highly dilute unfractionated cytosol, we conclude that NIF-2 activity is not already present at high levels in the material precipitated by 40% saturated ammonium sulfate. We were also unable to detect NIF-2 in the 40% ammonium-sulfate-soluble fraction of cytosol. Thus, NIF-2 appears to be inactivated by the salt treatment. From other experiments, we believe that NIF-2 is a macromolecule, since dialyzed crude cytosol had the same synergistic effect as seen in Fig. 5 (data not shown).

Discussion

This report has addressed the following question: are all the activities required for nuclear import contained in the nuclear pore, or are cytosolic factors also required? To answer

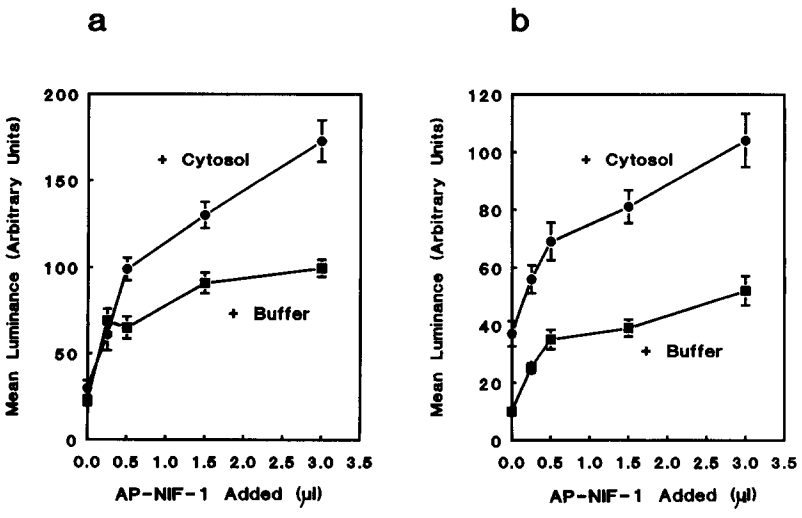

Figure 5. NIF-1 acts synergistically with a second factor present in untreated cytosol. The results of two separate experiments are shown in *a* and *b*. The experiments were performed essentially as in Fig. 3, except that the total assay volume was 1.5 times larger; i.e., for each assay sample, 9 µl of assay mixture (consisting of NEM-treated cytosol, untreated membranes, rat liver nuclei, and an ATP-regenerating system) was supplemented with the indicated amounts of AP-NIF-1, and then either 0.5 µl untreated cytosol (*solid circles*) or 0.5 µl buffer (*solid squares*) was added. The total volume of each sample was brought to 12.5 µl and 0.5 µl of a solution of the transport substrate, TRITC-HSA-wt, was added. After an incubation of 150 min (experiment in *a*) or 60 min (experiment in *b*), aliquots were removed and examined in the fluorescence microscope. Video image analysis (see Materials and Methods) was used to quantitate the fluorescence (luminance) of individual nuclei, in arbitrary units, reflecting the relative accumulation of TRITC-HSA-wt.

this question we made use of a cell-free system derived from an extract of *Xenopus* eggs. Previous studies have shown that this system displays all the hallmarks of authentic nuclear protein transport: specificity, requirement for an intact nuclear envelope, ATP- and temperature-dependence (Newmeyer et al., 1986*a*,*b*), inhibition by WGA (Finlay et al., 1987), and mediation by the nuclear pore (Newmeyer and Forbes, 1988). The egg extract is a complex mixture of macromolecules; nevertheless, our in vitro system opens the possibility of defining the minimum requirements for nuclear transport by means of biochemical fractionation. In particular, we have now found that isolated nuclei by themselves do not contain all the factors required for nuclear import (or at least not in sufficient quantity). Rather, at least two factors, which we have named NIF-1 and NIF-2, must be supplied by the cytoplasm. NIF-1 does not act simply to stabilize the nuclear envelope or to increase the number of functional nuclear pores but instead participates in the nuclear import mechanism per se.

Previously, we used the *Xenopus* in vitro system to show that the mechanism of nuclear import can be dissected experimentally into at least two steps: (*a*) ATP-independent binding at the nuclear pore; and (*b*) ATP-dependent translocation through the pore (Newmeyer and Forbes, 1988). Richardson et al. (1988) found similar results in vivo. In which step does NIF-1 participate? Our results show that NEM treatment of the cytosol inhibits signal-dependent nuclear pore binding, and that the addition of NIF-1 rescues binding activity. We conclude that NIF-1 is involved in the nuclear pore-binding step. However, since translocation of proteins through the pore cannot occur without prior binding of the protein to the pore, we cannot determine whether NIF-1 also participates in the translocation step. Further purification of the AP-NIF-1 fraction (now in progress) may reveal whether it contains a single factor or a set of NEM-sensitive macromolecules that are perhaps involved in more than one step of the transport reaction.

It should be noted that our results are in disagreement with two earlier reports that isolated nuclei, placed in defined buffers without added cytosol, may be functional in nuclear import (Markland et al., 1987; Imamoto-Sonobe et al., 1988). However, for neither of these simple systems is there electron microscopic evidence of protein translocation through the nuclear pores; those results must therefore be weighed cautiously. Although Markland et al. (1987) did observe ATP-dependent nuclear association, the effects of ATP-depletion were irreversible. Moreover, they did not measure the efficiency of the reaction; their results may have reflected a small amount of residual transport activity because of cytoplasmic contamination of the nuclei.

What precise function might NIF-1 serve? Because NIF-1 is required for signal-mediated binding to the pore, one hypothesis is that NIF-1 is a cytoplasmic carrier protein that recognizes the nuclear localization signal peptide; after binding the protein to be transported, NIF-1 would interact in turn with its own receptor at the nuclear pore. Adam et al. (1989), using chemical cross-linking methods, have identified proteins (60 and 70 kD in size) that bind the SV40 T-antigen nuclear localization signal in vitro. These proteins were present in both cytosolic and nuclear fractions. Yamasaki et al. (1989) also identified signal-binding proteins using a cross-linking assay. Two of these proteins, of molecular masses 55 and 140 kD, were found in rat liver nuclei. However, two others, 70 and 100 kD in size, were found in the cytosol from Buffalo rat liver cells. Using a different approach, Yoneda et al. (1988) identified two candidate receptor polypeptides (59 and 69 kD) that were detected only in the nucleus. These observations of signal-binding proteins located in both nucleus and cytoplasm are consistent with the idea of a shuttling carrier, although it has not yet been demonstrated that these proteins function as signal-receptors in vivo. If NIF-1 is a shuttling receptor, it may prove to be identical to one or more of these proteins. Our finding that nuclei in NEM-treated extracts cannot import proteins argues that NIF-1 activity is not present in assayable quantities in isolated rat liver nuclei. However, NIF-1 may be loosely or reversibly bound to the nuclear pore in situ but easily dissociated from the pore during our nuclear isolation procedure. If NIF-1 is a shuttling receptor, it would be only transiently associated with the pore; some fraction of the pool of NIF-1 should be cytoplasmic. The proportions of nuclear and cytoplasmic NIF-1 in this case would depend on the total amount of NIF-1 present in the cell. For example, in somatic cells, it might be that the total pool of NIF-1 is small, and therefore most of the NIF-1 might be loosely associated with the nuclear pore, while in *Xenopus* oocytes or eggs, NIF-1 may be stored in large quantities in the cytoplasm. In any case, our results are not inconsistent with the possibility that NIF-1 is a signal receptor, perhaps identical to, or containing, one of the signal-binding proteins previously identified.

There are, however, other possible ways in which a cytoplasmic factor might function in nuclear import. At present, we know little about the transport mechanism. Nevertheless, one plausible model based on electron microscopic data can be proposed. Fibers 3–4 nm in diameter have been observed emanating from the nuclear pores of nuclear envelopes manually isolated from *Xenopus* oocytes (Franke, 1970; Franke and Scheer, 1970; Franke et al., 1981). Richardson et al. (1988) found that nucleoplasmin-coated gold particles injected into the oocyte bind to these fibers. While we do not see nuclear pore-associated fibrils in our in vitro system, we nevertheless find that the signal-sequence binding sites can lie at considerable distance from the center of the pore (Newmeyer and Forbes, 1988). Such results suggest that pore fibrils may be involved in the transport mechanism. If the translocation motor does involve fibers and is analogous to one of the known fiber-motility systems in the cytoplasm (e.g., the kinesin/microtubule [Vale, 1987] or actin/myosin [Warrick and Spudich, 1987] systems), then one would expect to find a recycling component of the translocation motor, either the fiber monomer or a molecule moving along the fiber. NIF-1 and NIF-2 could be components of such a motility system. It is equally possible that NIF-1 and NIF-2 are neither signal sequence receptors nor proteins associated with fibers, but instead soluble factors (e.g., kinases) that activate a distinct signal-receptor molecule or interact with the signal-bearing protein to permit it to bind to the receptor. Future studies will take advantage of the *Xenopus* egg extract system to reconstitute the nuclear import machinery in vitro from purified factors like NIF-1 and NIF-2 and to study the roles of these factors in the transport mechanism.

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