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Reconstitution of Biochemically Altered Nuclear Pores: Transport Can Be Eliminated and Restored

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Summary

Biochemically altered nuclear pores specifically lacking the N-acetylglucosamine-bearing pore proteins were constructed in a nuclear assembly extract in order to assign function to these proteins. The depleted pores do not bind nuclear signal sequences or actively import nuclear proteins, but they are functional for diffusion. These defects can be fully repaired by assembly with readded Xenopus pore glycoproteins. Strikingly, isolated rat pore glycoproteins also restore transport. Electron microscopy reveals that depleted pores have largely normal morphology. Thus, the pore glycoproteins are not required for assembly of the nuclear envelope, the major structures of the pore, or a pore diffusional channel. Instead, they are essential for active protein import and, unexpectedly, for construction of the part of the pore necessary for signal sequence recognition.

Introduction

In eukaryotic cells, the cytoplasm and nucleus are divided into discrete compartments by the nuclear envelope. It is known that communication between these microenvironments occurs via nuclear pores. Nuclear transport is distinct from other transport systems in two respects. First, selective transport through the pores is a bidirectional process: RNA and preribosomes exit the nucleus, while karyophilic proteins enter. Second, the diverse nature of the transport materials requires that the nuclear pores be able to transport in a selective manner substrates ranging in size from 40–2000 kd while still providing open channels for the passive diffusion of smaller molecules. Currently, very little is known about the proteins responsible for regulating this uniquely flexible transport apparatus.

Electron microscopic studies have provided a model of the pore at 90 Å resolution. The pore consists of two outer rings of eight subunits, with one ring on either side of the nuclear envelope. Each subunit or annular granule appears connected to an inner ring of spokes that surround a central channel (Unwin and Milligan, 1982; Stewart and Whytock, 1988; Akey, 1989). Occasionally, fibers can be seen extending from the pore into the cytoplasm or the nucleus, possibly acting as tracks through the pore (Carmo-Fonseca et al., 1987; Georgatos and Blobel, 1987; Richardson et al., 1988; Scheer et al., 1988).

The resting diameter of the nuclear pore, ~90 Å, is such that it allows small proteins and metabolites to diffuse freely into the nucleus (<20–60 kd; Bonner, 1978; Paine and Horowitz, 1980; Peters et al., 1986; Peters, 1986; Lang et al., 1986; Lanford et al., 1986). Large nuclear proteins, however, must possess a nuclear signal sequence for transport through the pore (see Dingwall and Laskey, 1986, for review). The conjugation of a chemically synthesized nuclear sequence to a nonnuclear protein is sufficient to confer nuclear localization (Goldfarb et al., 1986; Lanford et al., 1986; Yoneda et al., 1987b; Newmeyer and Forbes, 1988; Dworetzky et al., 1988; Wolff et al., 1988). In response to a signal sequence, the resting pore channel appears to expand in diameter up to 260 Å to allow the entry of signal sequence–bearing proteins (Feldherr et al., 1984; Dworetzky et al., 1988; Dworetzky and Feldherr, 1988). It is unknown whether a specific signal sequence is also required for the exit of RNAs.

The development of an in vitro nuclear transport system has provided a readily accessible experimental system to probe pore function. When isolated nuclei are placed in a cytoplasmic extract derived from Xenopus eggs, the extract heals and/or maintains the nuclear envelopes in an intact state. When fluorescent- or gold-tagged proteins containing nuclear signal sequences are added, the proteins are transported and accumulate to high levels within the nuclei in an ATP-dependent manner (Newmeyer et al., 1986a, 1986b; Dreyer et al., 1986; Dreyer, 1987; Newmeyer and Forbes, 1988). In this system, it has been demonstrated that nuclear transport involves at least two steps: a step in which a nuclear protein binds to the pore in a signal sequence-dependent manner, and a subsequent but separate step in which the protein translocates through the pore (Newmeyer and Forbes, 1988). ATP is not required for the binding step, but it is required for the translocation step. Identical steps have also been observed in vivo (Richardson et al., 1988).

Analysis of nuclear transport in vitro identified the lectin wheat germ agglutinin (WGA) as a strong inhibitor of nuclear protein import (Finlay et al., 1987). The inhibitor WGA binds specifically to the nuclear pore (Finlay et al., 1987). WGA does not act by physically occluding the pore, since small fluorescent dextrans continue to diffuse at a normal rate into the nucleus (Finlay et al., 1987; Yoneda et al., 1987a; Dabauvalle et al., 1988a; Wolff et al., 1988). In a normal transport assay, gold particles coated with the SV40 large T antigen signal sequence are transported through the pore in large numbers (Feldherr et al., 1984; Dworetzky et al., 1988; Newmeyer and Forbes, 1988). When WGA is added, however, no translocation is observed. Instead, signal sequence–coated particles bind to the pore (Newmeyer and Forbes, 1988). Thus, in normal pores WGA specifically blocks the translocation step of transport but does not affect the signal sequence–dependent binding step.

The nuclear pore itself is a very large structure with an estimated mass of ~100 million daltons (Krohne et al., 1978; Blobel, 1985). To date, relatively few of the proteins that compose the pore have been identified. One protein, gp190, is an integral membrane glycoprotein containing a complex carbohydrate sidechain and has been proposed to anchor the pore within the nuclear membranes (Gerace et al., 1982; Filson et al., 1985). Eight to ten proteins, each

bearing an unusual carbohydrate modification consisting of single O-linked residues of N-acetylglucosamine (GlcNAc), have recently been identified as pore proteins (for review, see Hart et al., 1989). This family of proteins was identified by a number of anti-pore antibodies (Davis and Blobel, 1986, 1987; Snow et al., 1987; Holt et al., 1987; Park et al., 1987; Schindler et al., 1987; Hanover et al., 1987; Featherstone et al., 1988; Dabauvalle et al., 1988b). Such antibodies recognize single O-linked N-acetylglucosamine residues as part of their epitope (Holt and Hart, 1986; Holt et al., 1987; Hart et al., 1988). By gel electrophoresis and blotting with ^{125}I-WGA, an overlapping family of ~8–10 glycoproteins that binds the lectin WGA has been similarly identified in rat liver nuclei. The most prominent such protein in rat liver nuclei is a 62 kd glycoprotein (Finlay et al., 1987), identical in size to a 62 kd pore glycoprotein recognized by the anti-pore monoclonal antibody MAb 414 (Davis and Blobel, 1986, 1987). The inhibitory action of WGA thus appears quite explicable; i.e., WGA binds to pore proteins by binding to their N-acetylglucosamine residues, and in doing so blocks the translocation mechanism. A subset of the anti-pore antibodies also similarly inhibits transport when tested in vivo (Featherstone et al., 1988; Dabauvalle et al., 1988b). Biochemical analysis of these GlcNAc-bearing pore proteins has greatly expanded the potential candidates for proteins involved in pore function, although the sum of these proteins comprises only 20% of the proteins of the pore (Snow et al., 1987).

The study of nuclear transport has advanced to the point that one would like to assign specific functions to individual pore proteins. One approach would be to modify or remove a single pore protein and determine the consequence for pore function. The application of this strategy to the preexisting pores of rat liver nuclei is experimentally difficult. Similarly, a genetic approach has not yet been feasible. However, it is theoretically possible to accomplish a similar end by using a system that reconstitutes nuclei and nuclear pores de novo. Our strategy has been to deplete the entire family of WGA binding pore proteins from a nuclear assembly extract and to reconstitute nuclei lacking these pore proteins. We find that these nuclei are defective for nuclear transport. Pores formed in the absence of these proteins are structurally very similar to normal pores and continue to carry out one pore function, the passive diffusion of small molecules. The pores are specifically altered, however, in active transport of signal sequence–bearing proteins and are incapable of the first step in transport, binding to the pore. Thus, we have found it possible to create nuclei with biochemically altered pores and to test the functional consequences of specific alterations in pore protein content.

Results

Xenopus Egg Extracts Contain N-Acetylglucosamine-Bearing Pore Proteins in a Soluble Form

To determine whether nuclei could be formed containing pores depleted of a specific class of pore glycoproteins,

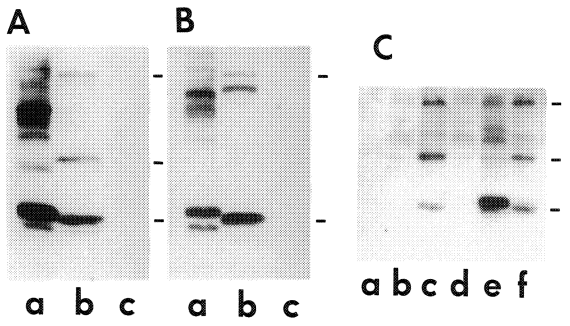

Figure 1. Xenopus Egg Cytosol Contains WGA Binding Pore Proteins

(A) Xenopus egg cytosol contains proteins that bind WGA. Rat liver nuclei (lane a; 3 × 10^6), the soluble fraction of Xenopus egg extracts (lane b; 10 μl), and the membrane fraction of the egg extract (lane c; 1 μl of a 10× concentrated fraction) were electrophoresed on 10% SDS–polyacrylamide gels, transferred to nitrocellulose, and probed with ^{125}I-WGA. Bars indicate the 60, 97, and 200 kd Xenopus WGA binding proteins seen in all blots.

(B) Xenopus egg proteins cross-react with an antipore antibody. Proteins derived from rat liver nuclei (lane a; 1.5 × 10^6), Xenopus cytosol (lane b; 10 μl), or membranes (lane c; 1 μl of a 10× concentrated fraction) were electrophoresed, transferred to nitrocellulose, and probed with the rat antipore monoclonal antibody MAb 414. Prominent Xenopus proteins of 200, 180, and 60 kd are seen to cross-react with MAb 414. Bars mark the 60 and 200 kd Xenopus proteins.

(C) Three soluble Xenopus WGA binding proteins pellet after nuclear formation. Sperm chromatin (10,000/μl) was added to cytosol plus membranes and incubated in the presence of ATP for 120 min. The nuclei formed were pelleted as described in Experimental Procedures (lane c). In parallel experiments, chromatin, membranes, and ATP were added to cytosol depleted for the WGA binding proteins (lane d), depleted cytosol to which isolated rat nuclear WGA binding proteins had been added (lane e), or depleted cytosol to which Xenopus WGA binding proteins had been added (lane f). In lane a, sperm chromatin alone was electrophoresed and probed with ^{125}I-WGA. In lane b, membranes and soluble fractions were incubated with ATP in the absence of any chromatin and treated identically to the other fractions. The soluble Xenopus 60, 97, and 200 kd proteins become pelletable only when membranes, cytosol, and chromatin are present (lanes c and f). Rat pore proteins also pellet upon nuclear formation (lane e). Bars are as in Figure 1A.

we used a nuclear reconstitution system derived from Xenopus eggs. Normally, such a reconstitution extract is able to assemble nuclei de novo when DNA or chromatin is added (Forbes et al., 1983; Lohka and Masui, 1983, 1984; Newport and Forbes, 1985; Newmeyer et al., 1986a; Blow and Laskey, 1986; Dreyer et al., 1986; Newport, 1987; Sheehan et al., 1988). The normal reconstituted nuclei contain double nuclear membranes, a lamina, and numerous nuclear pores and are capable of efficient replication and nuclear transport. The reconstitution extract can be further separated into soluble and membrane fractions by high speed centrifugation, both of which are required for nuclear assembly (Lohka and Masui, 1983, 1984; Newport, 1987; Sheehan et al., 1988; Wilson and Newport, 1988).

To use this extract to construct depleted pores, it was necessary first to ask whether the Xenopus extract contains N-acetylglucosamine-bearing pore proteins homologous to those of rat liver nuclei. Hence, we separated the reconstitution extract into soluble and membrane frac-

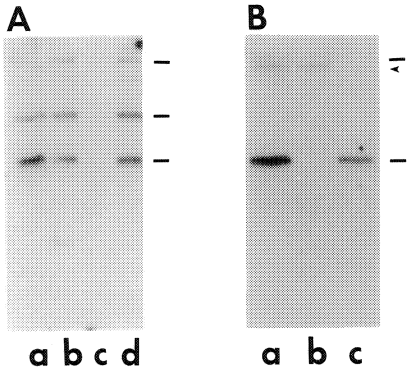

Figure 2. Xenopus Egg Cytosol Can Be Depleted of WGA Binding Pore Proteins

(A) The following fractions were electrophoresed, blotted, and probed with ^{125}I-WGA: cytosol applied to unconjugated Sepharose (lane a; 5 μl), cytosol applied to WGA-Sepharose in the presence of competing sugar (lane b; 5 μl), and cytosol applied to WGA-Sepharose (lane c; 5 μl, depleted). The depleted extracts were routinely determined to be 95%–100% depleted of WGA binding pore proteins. The Xenopus proteins bound by WGA-Sepharose could be recovered by elution with competing sugar (lane d; proteins eluted from WGA beads; 5 μl). Bars mark the 200, 97, and 60 kd bands.

(B) Undepleted (lane a) and depleted (lane b) egg extracts (10 μl) were electrophoresed, blotted to nitrocellulose, and probed with MAb 414. The WGA binding proteins eluted from WGA-Sepharose were treated in the same manner and probed with MAb 414 (lane c). The depleted extract is seen to lack the 200 and 60 kd Xenopus WGA binding proteins that cross-react with MAb 414. Bars mark the 200 and 60 kd bands; an arrowhead marks the 180 kd band.

tions. Each fraction was analyzed by gel electrophoresis, blotted onto nitrocellulose, and probed with ^{125}I-labeled WGA. We found that the soluble fraction of the Xenopus extract contained three major WGA binding proteins with molecular masses of 200, 97, and 60 kd (Figure 1A, lane b). Minor protein species were variably present. The membrane fraction, however, contained no abundant WGA binding glycoproteins (Figure 1A, lane c).

To determine whether the soluble Xenopus WGA binding proteins are pore protein homologs, the proteins were tested for cross-reactivity with a monoclonal anti-pore antibody, MAb 414. This antibody binds specifically to the rat pore proteins p62, p180, and p270 as a result of a shared epitope (Davis and Blobel, 1987). MAb 414 recognized three prominent proteins in the Xenopus extract: the 60 and 200 kd proteins and a third protein of ~180 kd (Figure 1B, lane b). We conclude that the 60 and 200 kd proteins are Xenopus pore proteins. Because the 180 kd protein cross-reacts with MAb 414 but not with WGA, we think it is also a Xenopus pore-related protein lacking the clustered GlcNAc residues required for WGA binding. This is supported by the finding that a 180 kd protein is a very prominent pore protein in rat nuclei (Figure 1B, lane a; Snow et al., 1987). Thus, Xenopus eggs do contain WGA binding pore protein homologs, and the majority appear to be stored in a soluble form in the egg cytoplasm.

One would predict that the initially soluble Xenopus pore proteins would become incorporated into nuclei during the course of nuclear assembly in cell-free extracts. To test this prediction, sperm chromatin was added to a mixture of soluble and membrane components to allow nuclear assembly. The newly formed nuclei were pelleted, and proteins were extracted, fractionated by electrophoresis, and assayed for WGA binding glycoproteins on blots probed with ^{125}I-WGA. We found that each of the three major WGA binding proteins present in the soluble fraction (200, 97, and 60 kd) pelleted with the newly formed nuclei (Figure 1C, lane c). Sperm chromatin alone contained no observable WGA binding proteins (Figure 1C, lane a). As a control, sperm chromatin was added to the soluble fraction in the absence of membranes and then pelleted; in this case, no WGA binding proteins pelleted (Figure 1C, lane b). We conclude that the three soluble WGA binding proteins do become incorporated into nuclei and, furthermore, that this incorporation requires the presence of membranes.

Nuclei with Intact Nuclear Envelopes Can Form in the Absence of WGA Binding Pore Proteins

Having established that the Xenopus extract contains WGA binding pore protein homologs, we attempted to prepare a nuclear reconstitution extract lacking these proteins. The soluble fraction of an egg extract was chromatographed on WGA-Sepharose. By this treatment, nuclear reconstitution extracts were found to be quantitatively depleted of all three WGA binding pore proteins, as shown by blotting with ^{125}I-labeled WGA (Figure 2A, lane c) or MAb 414 (Figure 2B, lane b, for the 60 and 200 kd proteins). The 180 kd protein, which does not bind to WGA, was not depleted.

We asked whether the depleted extracts were capable of forming nuclei upon addition of chromatin and membranes, as measured by the acquisition of an intact nuclear envelope. Presence of an intact envelope was assessed by the possession of a phase-dense rim around each nucleus (data not shown) and, more definitively, by the ability to exclude large fluoresceinated dextrans (150 kd; Figures 3b and 3d). We found that nuclei with intact nuclear membranes did form in depleted extracts (Figure 3b), just as they did in control extracts (Figure 3d). We conclude that the majority of WGA binding pore proteins in the extract are not required for the formation of the nuclear envelope.

Nuclei That Lack the WGA Binding Pore Proteins Are Defective for Transport

To ask whether nuclei depleted of the WGA binding pore proteins were capable of transport, a fluorescent nuclear transport substrate was added 90–180 min after nuclear formation. The substrate consisted of rhodamine-labeled human serum albumin (HSA) covalently coupled to multiple copies of a nuclear signal sequence peptide (designated TRITC-ss-HSA; Newmeyer and Forbes, 1988). The nine amino acid signal sequence peptide corresponded to the transport signal of the SV40 large T antigen (Kalderon et al., 1984a, 1984b; Lanford and Butel, 1984; Goldfarb et al., 1986) and was synthesized chemically before coupling to HSA. Previous experiments es-

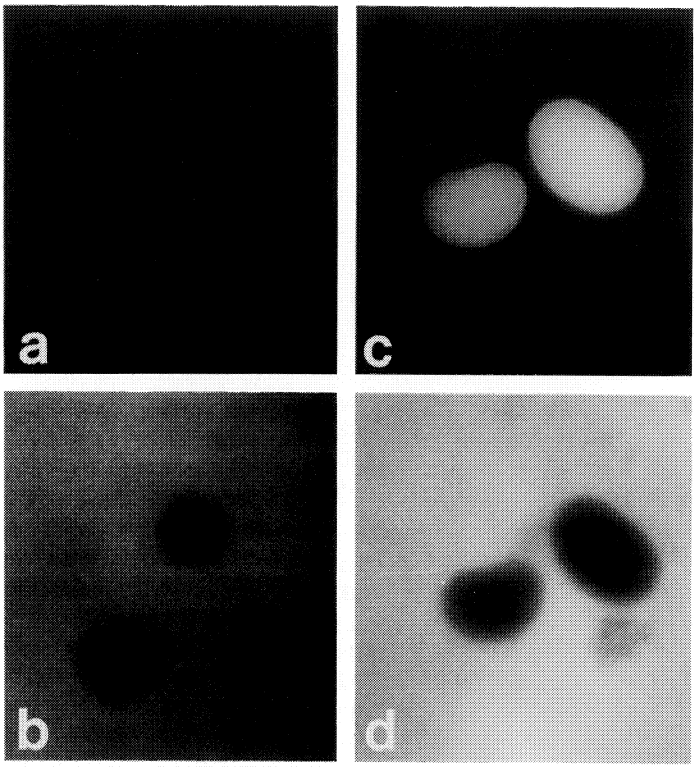

Figure 3. Nuclei Form in Depleted Extracts but Do Not Transport

Sperm chromatin was added to a depleted (a and b) or control extract (c and d) to which membranes and an ATP-regenerating system were then added (see Experimental Procedures). (The control extract was prepared by applying extract to a WGA-Sepharose column in the presence of competing sugar.) Subsequent nuclear formation was assayed by the ability to exclude FITC-labeled 150 kd dextran; both control (d) and depleted nuclei (b) exclude FITC-dextran. Ten minutes after the majority of nuclei were observed to be excluding 150 kd FITC-dextran, RITC-ss-HSA transport substrate was added, and transport was assayed 80 min later with the fluorescence microscope. Control nuclei showed normal transport (c), while depleted nuclei showed no transport (a).

tablished TRITC-wt-HSA as a very efficient and specific transport substrate (Newmeyer and Forbes, 1988).

We found that nuclei formed in a control extract (applied to unconjugated Sepharose) transported TRITC-ss-HSA to high levels at 80 min (Figure 3c). In contrast, nuclei formed in a depleted extract showed little or no accumulation of the transport substrate at 80 min (Figure 3a). Only nuclei capable of excluding large FITC-dextrans were assayed for accumulation. In a typical experiment, 98%–100% of the depleted nuclei that excluded the 150 kd FITC-dextrans showed no accumulation of the nuclear transport substrate. It should be noted, however, that if the depleted nuclei were incubated for long periods (>3 hr) at normal substrate concentrations (1.25–2.5 μg/ml) or, alternatively, if extremely high levels of transport substrate were added, some low level of accumulation of TRITC-ss-HSA could be observed (data not shown). Precise quantitation of transport in depleted nuclei with a fluorescence microfluorimeter indicated a large decrease in the level of transport (≤3% of control, see below) and in the number of depleted nuclei capable of low levels of transport. We conclude that in removing the WGA binding proteins we remove components essential for nuclear transport.

Transport Competence Is Restored by Addition of the WGA Binding Pore Proteins

To determine whether the defect in transport was specifically caused by removal of the WGA binding proteins, we asked whether transport function was restored by adding back the Xenopus WGA binding pore proteins to a depleted extract. To isolate these proteins, the soluble egg extract was applied to WGA-Sepharose beads, and the beads were washed extensively with buffer to remove nonspecifically bound proteins. The Xenopus WGA binding proteins were eluted with the competing sugars N-acetylglucosamine (125 mM) and N,N′,N″-triacetyl-chitotriose (2 mM) into one-fourth the original volume, concentrating the proteins ~4-fold. This procedure resulted in the quantitative recovery of the proteins, as determined by gel electrophoresis and blotting with ^{125}I-labeled WGA (Figure 2A, lane d). When the eluted proteins were added to a nuclear reconstitution assay, all three proteins were found to pellet with the newly formed nuclei (Figure 1C, lane f).

To test whether transport was restored, the WGA binding proteins were added to a depleted extract in the presence of membranes, sperm chromatin, and an ATP-regenerating system. After incubation for >90 min to allow for nuclear assembly, TRITC-ss-HSA was added and the reconstituted nuclei were monitored for transport by fluorescence microscopy 20–180 min later. We found that addition of the Xenopus WGA binding proteins completely restored the ability of the nuclei to transport (Figures 4b and 4d). The level of accumulation was typical of control extracts, while no accumulation was found in the depleted extract (Figures 4a and 4c).

One would predict that it might also be possible to add these proteins to depleted nuclei *after* the nuclei are formed. Preliminary experiments indicate that this is the case (data not shown). However, because of the capacity of the extract to form nuclei throughout the course of the reaction, it is possible that the WGA binding pore proteins, when added at late times, associate with a small fraction

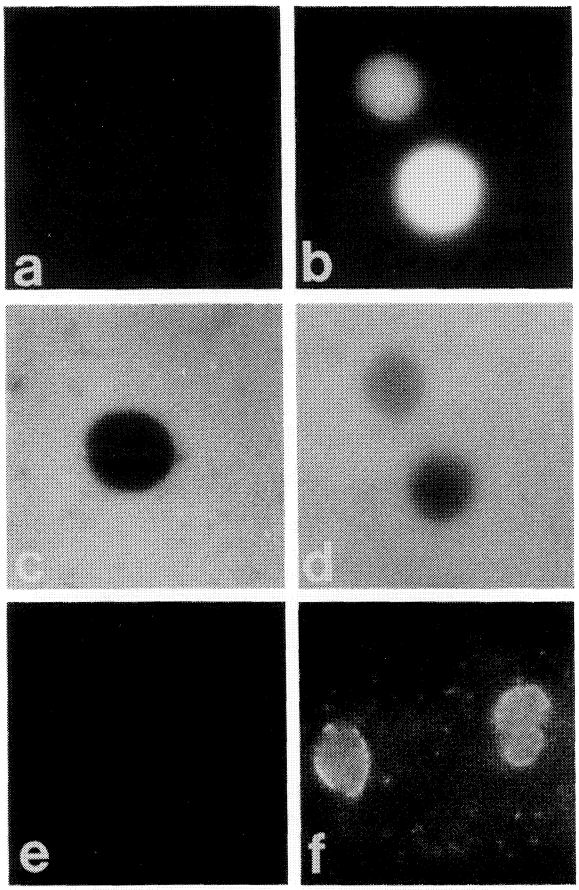

Figure 4. Addition of Xenopus WGA Binding Proteins to a Depleted Extract Restores the Ability to Form Transport-Competent Nuclei

Sperm chromatin was added to depleted extracts (a, c, and e) or depleted extracts supplemented with Xenopus WGA binding proteins (b, d, and f). Both were then mixed with membranes and an ATP-regenerating system. Nuclear formation and transport were assayed as in Figure 3. The nuclei that formed in depleted extracts (c) or depleted extracts supplemented with Xenopus WGA binding proteins (d) were observed to exclude FITC-150 kd dextrans. Transport was negative in depleted nuclei (a) but restored in nuclei containing exogenously added Xenopus WGA binding proteins (b). Binding of RITC-ss-HSA to the pore in the absence of ATP was assayed as described in Experimental Procedures. In the absence of ATP, depleted nuclei showed no binding at 60 min (e), while typical nuclear rim binding was seen with nuclei containing added Xenopus WGA binding proteins (f).

of unhealed or unformed nuclei and are incorporated into pores de novo. Since such late add-back experiments are subject to multiple interpretations, we have not concentrated on them here.

To quantitate the depletion and add-back results precisely, a fluorescence-activated microfluorimeter (FMF) technique was used, capable of assaying thousands of individual nuclei for the amount of fluorescently labeled nuclear protein accumulated in each (Finlay et al., 1989; Hartl and Forbes, unpublished data). The results of several experiments are presented in Table 1. The majority of control nuclei are observed to transport and accumulate high levels of fluorescent substrate (5–100 U). Most of the depleted nuclei, however, have fluorescence values equivalent to the background level seen in the absence of any FITC-ss-HSA (0–2 U). Even when the depleted nuclei do show some FITC-ss-HSA association, this level is much lower (2–4.9 U). Analysis of the data shows that total transport in depleted nuclei is 2%–3% of control values (Table 1). In contrast, when nuclei are formed in depleted extracts to which the Xenopus proteins were readded, transport is restored to high levels (142% of control, Table 1, Experiment 3). Thus, the FMF results confirm the visual results that active transport is virtually eliminated in the absence of the WGA binding pore proteins and restored by the addition of these proteins.

The WGA Binding Pore Proteins Are Required to Construct the Binding Portion of the Pore

It was previously demonstrated that nuclear transport can be experimentally divided into two steps: pore binding and translocation through the pore (Newmeyer and Forbes, 1988; Richardson et al., 1988). Such pore binding can be visualized as a punctate fluorescent nuclear rim stain owing to the presence of fluorescent transport substrate bound to pores or, in the electron microscope, as signal sequence–coated gold particles bound to the cytoplasmic sides of the nuclear pores (Newmeyer and Forbes, 1988). To assay for pore binding in depleted and control nuclei, the transport reaction was performed in the absence of ATP (Figures 4e and 4f) to prevent translocation into the nucleus. We found that no pore binding of TRITC-ss-HSA occurred in nuclei lacking the pore glycoproteins, i.e., no fluorescent rim indicative of pore binding was seen in depleted nuclei (Figure 4e). However, binding and translocation were restored when the Xenopus WGA binding pore proteins were added back to the depleted extract (Figures 4b and 4f). These results demonstrate that one or more of these pore proteins is essential for the signal sequence–dependent binding of nuclear proteins to the pore. Thus, at a functional level, removal of the WGA binding proteins eliminates the binding step of transport, and addition of the WGA binding proteins effectively restores binding.

Rat Pore Proteins Restore Transport to Nuclei Lacking the Equivalent Xenopus Pore Proteins

The lectin WGA was first observed to inhibit nuclear transport in rat liver nuclei (Finlay et al., 1987). Moreover, biochemical analyses of the WGA binding pore proteins have focused primarily on the proteins of rat liver nuclei (Davis and Blobel, 1986, 1987; Finlay et al., 1987; Snow et al., 1987; Holt et al., 1987; Park et al., 1987; Schindler et al., 1987; Hanover et al., 1987). We wished to determine whether rat nuclear pore glycoproteins could functionally substitute for the Xenopus WGA binding pore proteins. To obtain the rat pore glycoproteins, rat liver nuclei were incubated with the detergent Mega 10 (2%), which extracts nuclear membrane proteins and a large fraction of WGA binding proteins from the pores (Finlay et al., 1987; Horecka and Forbes, unpublished data). The detergent extract was

Table 1. Quantitation of Nuclear Transport in Control, Depleted, and Reconstituted Nuclei Using the Fluorescence Microfluorimeter

	Number of Nuclei Containing X Units of Accumulated Transport Probe					Total Nuclear Accumulation
	0–2 U	2–4.9 U	5–24.9 U	25–44.9 U	45–100 U	(% of Control)
Experiment 1						
Control	1,797	5,405	8,155	608	144	100
Depleted	14,255	1,213	73	0	0	3.2
Experiment 2						
Control	837	1,179	4,049	1,954	2,687	100
Depleted	9,904	640	167	10	0	2
Experiment 3						
Control	4,959	5,542	6,072	99	0	100
Depleted	16,366	321	78	2	0	2.1
+ Xenopus	3,263	5,050	7,507	767	117	142

Nuclear transport was assayed in large numbers of individual nuclei with the fluorescence microfluorimeter, as described in Experimental Procedures. The number of nuclei showing a given range of transport (for example, 0–2 transport units) was pooled from a printout of the raw data. Values of 0–2 do not represent transport, because they were routinely obtained even in the absence of FITC-ss-HSA as a result of bleedthrough from the propidium iodide channel into the FITC channel.

The total number of nuclei monitored in Experiments 1, 2, and 3, respectively, was ~16,000, 10,800, and 16,800. Fluorescence units are arbitrary units set by the microfluorimeter. Total nuclear accumulation was calculated for each experimental aliquot by multiplying the number of nuclei in each category by the mean fluorescence units in that category. For example, in the Control sample Experiment 1, total accumulated fluorescence was calculated to be (5405 × 3.5) + (8155 × 15) + (608 × 35) + (144 × 45) = 169,002 U. Total accumulation in depleted nuclei in Experiment 1 was calculated to be (1213 × 3.5) + (73 × 15) = 5340 U. The Depleted was thus 3.2% of the Control. It should be noted that in all cases where a sample contained nuclei in the 45–100 U category, the number of nuclei was multiplied by 45, thus giving a minimum estimate of total fluorescence in this category.

applied to WGA-Sepharose beads, and after extensive washing the bound rat WGA binding pore proteins were eluted with N-acetylglucosamine and trichitotriose (Figure 5, lane c). As controls, an equal volume of detergent extract was applied to unconjugated Sepharose (Figure 5, lane d) or to WGA-Sepharose plus competing sugar (data not shown), and the Sepharose was washed and mock eluted with identical sugar concentrations (designated "O-beads control eluate").

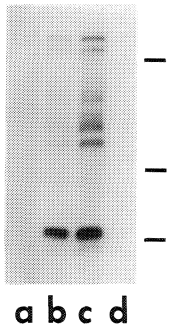

Figure 5. Isolation of Rat WGA Binding Pore Proteins

Rat WGA binding pore proteins were extracted from nuclei with the detergent Mega-10 and isolated using a WGA-Sepharose column. Shown are 5 μl of rat proteins after elution from the column with competing sugar (lane c) or after elution with sugar and subsequent dialysis (lane b). Rat nuclear proteins (5 μl) applied to an unconjugated Sepharose column and mock-eluted are shown in lane d. Shown for comparison is the depleted extract after application to a WGA-Sepharose column (lane a; 14 μl). The gel was blotted to nitrocellulose and probed with ^{125}I-labeled WGA. For size reference, bars mark the positions of migration of the Xenopus 60, 97, and 200 kd proteins in a parallel gel.

Five microliters of partially purified rat WGA binding proteins (Figure 5, lane c) was added to 15 μl of depleted Xenopus extract (Figure 5, lane a), followed by the addition of sperm chromatin and membranes. After 1–3 hr, the mixture was examined by fluorescence microscopy. We observed that normal nuclei formed both with the O-beads control eluate and with the added rat WGA binding proteins. The nuclei were intact since they were capable of excluding large fluorescent dextrans (Figures 6a–6c). When transport substrate was added to nuclei reconstituted in a depleted extract containing control eluate, no transport was seen (Figure 6d). In contrast, the nuclei reconstituted in a depleted extract plus added rat WGA binding pore proteins showed high levels of accumulation of the transport substrate (Figures 6e and 6f). Binding of the transport substrate to the nuclear envelope in the absence of ATP was also restored by addition of the rat WGA binding pore proteins (data not shown). When we asked which rat WGA binding proteins associate with nuclei during nuclear assembly, we found that a number of the rat pore proteins pelleted with the newly formed nuclei, including p62 (Figure 1C, lane e). In FMF experiments, the rat proteins restored transport to the high levels seen with the Xenopus proteins (data not shown). We conclude from these experiments that rat WGA binding pore proteins can effectively substitute for the missing Xenopus pore proteins and can completely restore transport competence.

The nuclei formed with rat pore proteins equalled or surpassed nuclei formed in undepleted extracts in transport capability. The minimum concentration of rat WGA binding proteins necessary to reconstitute normal levels of transport was found to be equivalent to the WGA binding proteins from ~8.4 × 10^5 rat liver nuclei (per 20 μl trans-

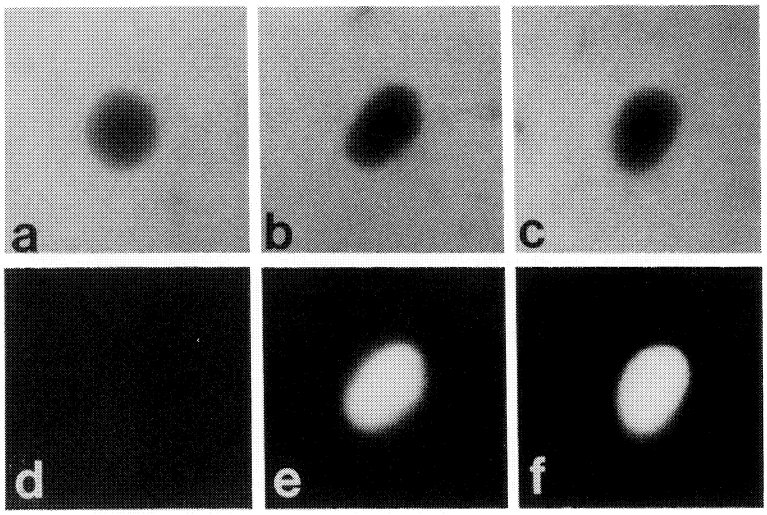

Figure 6. Rat Nuclear Pore Proteins Can Functionally Substitute for Xenopus Pore Proteins

Sperm chromatin was added to a depleted extract (a and d) or to a depleted extract supplemented with isolated rat nuclear WGA binding pore proteins (b, c, e, and f) before the addition of membranes and an ATP-regenerating system. Nuclei formed in both extracts excluded FITC-150 kd dextrans (a, b, and c). Transport was assayed as in Figure 3; lack of transport was observed with depleted nuclei (a). Nuclei containing rat WGA binding pore proteins showed high levels of transport 80 min after the addition of TRITC-ss-HSA (e and f).

port assay). Using the estimate from electron microscopy of 4000 pores/rat liver nucleus (Maul, 1977), the WGA binding proteins derived from $\sim 3.4 \times 10^9$ rat pores are being added to the depleted extract. From densitometric scanning of radiolabeled WGA and antibody blots (Finlay, Bradley, and Forbes, unpublished data), crude calculations allow one to estimate that we are adding rat proteins in ~1- to 2-fold excess of the amount of Xenopus pore proteins depleted from the extract (11.5 μl).

Nuclei Lacking the WGA Binding Pore Proteins Contain Visible Pores Capable of Diffusion

To determine the effect of removal of the WGA binding proteins on the structure of the nuclear envelope, aliquots of extracts containing depleted or control nuclei were fixed as in Sheehan et al. (1988), sectioned, and prepared for electron microscopy. Seven separate experiments were examined, and the nuclei formed in control and depleted extracts were compared. Nuclei formed in control extracts had nuclear envelopes containing numerous nuclear pores, as expected (data not shown). Nuclei formed in the depleted extracts, however, also contained visible nuclear pores in approximately similar numbers (Figure 7). The resolution of transmission electron microscopy on sectioned nuclei is limited in the number of pore substructures visible. Within this limited resolution, however, the pores of the depleted nuclei appeared identical in shape and size, although slightly less dense than normal pores. Pores formed in nuclei to which the proteins had been readded also appeared approximately identical to those of the control nuclei—within the structural resolution possible (data not shown).

Based on these observations, we conclude that the WGA binding pore proteins are essential for pore function

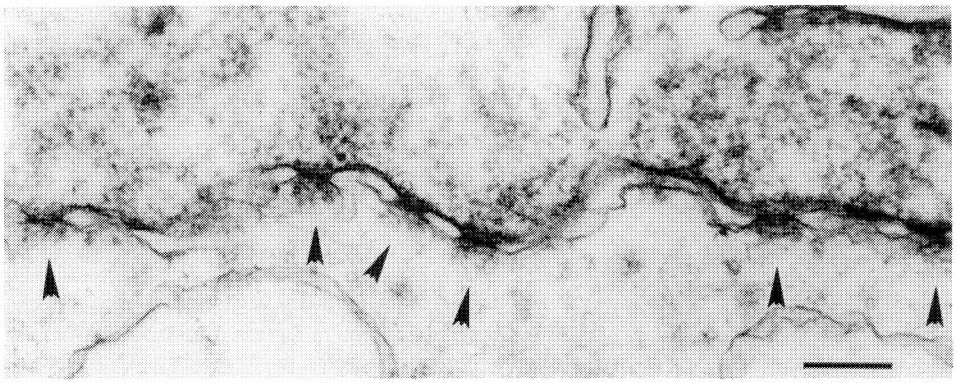

Figure 7. Depleted Nuclei Contain Nuclear Pores

Depleted nuclei were fixed, sectioned, and examined with the electron microscope as described in Experimental Procedures. A section of a depleted nucleus is shown. Although depleted nuclei varied in their content of pores, pores were commonly seen in depleted nuclei in seven independent experiments (arrowheads). In the experiment shown, no transport was observed with the depleted nuclei at 5 hr (= total incubation time; 1.5 hr after TRITC-ss-HSA addition), whereas high levels of transport were observed in control nuclei. In some experiments the depleted nuclei contained an excess of pores over the control, while in others they contained fewer pores. Because of the inherent variation in individual nuclei in any given assembly experiment, we cannot rule out the existence of a small but consistent difference in pore number or pore structure in the depleted nuclei. The only visible difference was that depleted pores appeared less dense than control pores. The bar represents 0.2 μm.

but that they appear not to comprise the major structural elements of the pore. Consistent with the majority of the structure being intact in depleted pores as seen in the electron microscope, we found that small 10 kd fluorescent dextrans passively diffused into these nuclei (data not shown), while large dextrans did not (see Figure 3b). Taken together, these results indicate that the passive channel of the pore is intact in depleted nuclei but that the signal-mediated opening of the pore to admit large nuclear proteins requires that the WGA binding pore proteins be present and active.

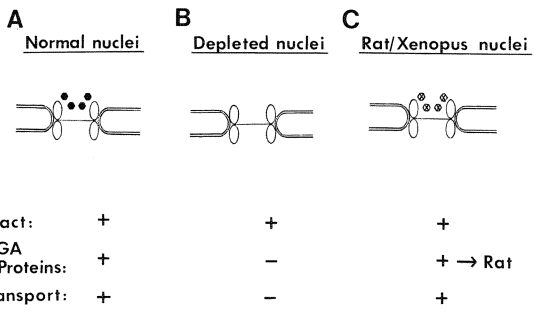

Figure 8. A Model for Nuclear Pore Structure in Normal and Depleted Nuclei

A simple model showing the proposed location of the Xenopus WGA binding proteins in the nuclear pore is presented (A: black hexagons). The ovals represent the annular granule subunits of the pore. Four are visible in this cross-section. It is proposed that the WGA binding proteins represent a subset of total pore proteins and lie at the periphery of the pore. In depleted nuclei, nuclear pores are detectable with the electron microscope but lack the WGA binding proteins (B) and have been shown to be nonfunctional for transport and binding. The WGA binding proteins derived either from Xenopus egg extracts (data not shown) or from rat liver nuclei (C: cross-hatched hexagons) when exogenously added to a depleted extract allow formation of transport-competent nuclei. In the case of added rat pore proteins, hybrid rat/Xenopus nuclear pores must form and are functional. The simplest model is shown; a model where the WGA binding proteins are present on both sides of the pore is equally consistent with the data.

Discussion

Recent work on the nuclear pore has centered on the search for pore proteins. This has been a difficult task both because of the very large size of the pore and because the pore cannot be easily purified away from the nuclear lamina and matrix. The isolation of monoclonal antibodies (Davis and Blobel, 1986, 1987; Snow et al., 1987; Holt et al., 1987; Park et al., 1987) and the discovery of an inhibitor of transport that bound to the pore (Finlay et al., 1987) led to the identification of a family of unusual cytoplasmically disposed glycosylated pore proteins (see Hart et al., 1989, for review). The inhibition of nuclear transport by WGA and by monoclonal antibodies whose epitope closely overlaps the binding site of WGA argued that one or more of these glycoproteins was critical to the overall function of the pore.

In this report, we demonstrate that the N-acetylglucosamine-bearing pore proteins can be dispensed with for many of the structures and functions of the pore. Specifically, these WGA binding pore proteins do not form the major visible structures of the pore, such as the bulk of the annular granules or pore diaphragm. They are not required for the assembly of the nuclear envelope, which might well be predicted to involve one or more pore proteins. Nor are the proteins required to form the channel of the pore used in passive diffusion. However, the N-glycosylated pore proteins are required for the active transport of nuclear proteins. Specifically, depleted pores are no longer able to bind proteins carrying a nuclear transport signal sequence. This is an unexpected result, since we previously showed that the lectin WGA, when added to normal pores, blocks translocation but has no effect on the binding step of transport. Our results indicate that the glycosylated pore proteins either are directly involved in the recognition of signal sequences at the pore or are a necessary structural component required for the assembly of proteins that mediate the recognition of transport substrates. The glycosylated pore proteins are also specifically required for the subsequent translocation step of active transport (Finlay et al., 1987; Newmeyer and Forbes, 1988).

Upon removal of the WGA binding pore proteins and formation of depleted nuclei, at least three possible outcomes were envisioned: absence of nuclear pores, visibly altered pores, or pores identical in structure and number to normal nuclei. We found that nuclear pores do form in the absence of WGA binding proteins. The pores in depleted nuclei appear normal, albeit less dense. Thus, any structural alterations of the pore that occur are beyond the limits of the resolution of our electron microscopy. The addition of isolated Xenopus WGA binding pore proteins to a depleted extract results in nuclei that are restored for both normal binding and transport. Moreover, the addition of the homologous pore proteins from rat liver nuclei similarly restores efficient transport. These data are incorporated into a simple model in Figure 8. In this model, the WGA binding pore proteins do not contribute all of the visible structure elements of the pore, but instead they are localized to the periphery. Nuclei formed in a depleted extract lack this outer layer of proteins. Pores reconstituted with either Xenopus or rat WGA binding proteins have this layer of proteins restored and are functional for transport. In the case of added rat proteins, the pores are actually of hybrid rat/Xenopus origin. It should be noted that the simplest model is drawn; a more symmetric model where WGA binding proteins are present peripherally on both sides of the pore is also consistent with the data. Thus, this pore reconstitution system has allowed us to demonstrate at the molecular level a specific role for the N-glycosylated pore proteins in selective transport of nuclear proteins through the pore, and, furthermore, an involvement in both the binding and translocation steps.

A peripheral location of WGA binding pore proteins is not inconsistent with the WGA inhibition data, since one or more such proteins must be accessible to the inhibitor (Finlay et al., 1987; Yoneda et al., 1987a; Dabauvalle et al., 1988a). A caveat of previous WGA- and antibody-binding electron microscopic studies performed on normal pores, however, has been that the observed location might be con-

strained by carbohydrate residue or antigen accessibility: i.e., binding of WGA or antibodies would only be observed at the periphery, but internally located glycoproteins vital for the existence of basic pore structures would not be visualized by these techniques (Nigg, 1988). Such electron microscopic binding studies indicated a peripheral location of these proteins in the region of the pore circumscribed by the annular granule subunits (identical to the area marked by black hexagons in Figure 8; Davis and Blobel, 1986, 1987; Finlay et al., 1987; Snow et al., 1987; Hanover et al., 1987; Park et al., 1987; Scheer et al., 1988; Dabauvalle et al., 1988b; Akey and Goldfarb, 1989). Our pore reconstitution results and electron microscopic studies indicate that the WGA binding proteins do appear to be confined to the periphery of the pore.

The nuclear pores depleted of WGA binding proteins are defective in signal sequence binding. As stated, this was unexpected, because in normal pores WGA does not block the binding step of transport but affects only the translocation step (Newmeyer and Forbes, 1988). It is possible that depleted pores lack binding because the signal sequence receptor is a WGA binding pore protein. A more likely conclusion may be that the receptor requires the presence of the WGA binding proteins to assemble onto the pore, if the receptor is a permanent component of the pore, or to dock at the pore, if the receptor shuttles between the nucleus and cytoplasm. Several groups have identified candidates for signal sequence receptors (Yoneda et al., 1988; Adam et al., 1989; Yamasaki et al., 1989; Silver et al., 1989). Determination of whether these contain N-acetylglucosamine residues awaits further work, but one report indicates that the signal sequence–binding proteins lack these residues (Yamasaki et al., 1989). The putative receptor proteins are found in both the cytoplasm and nucleus and thus may indeed be shuttling receptors (Adam et al., 1989; Yamasaki et al., 1989). The data presented here demonstrate that whatever the receptor, it requires the presence of the WGA binding proteins to dock at the pore.

From our results, rat pore glycoproteins are functionally homologous to Xenopus pore proteins. Other examples in which heterologous systems have been shown to complement are increasing in number. For example, yeast cytosolic transport factors can efficiently substitute for their mammalian counterparts in vesicular transport between successive Golgi compartments (Dunphy et al., 1986). Similarly, a protein (p13) involved in regulation of the yeast cell cycle interacts specifically with a component of the Xenopus cell cycle regulator MPF (maturation promoting factor; Dunphy et al., 1988; Gautier et al., 1988). In electron microscopic studies of many different organisms, the nuclear pore is quite conserved at the ultrastructural level. Antibodies to rat pore proteins cross-react with Xenopus proteins (see Figure 1B; Featherstone et al., 1988). The data presented here demonstrate that certain proteins that underlie these structural similarities are functionally interchangeable. These results suggest that cross-species combinations of pore proteins will be a powerful tool in correlating structure and function in the pore.

Relatively little is known about the way a nuclear pore is assembled at the end of mitosis. The in vitro nuclear reconstitution extracts described here are derived from eggs arrested in mitosis. During the preparation of the extracts, mitotic activities are inactivated, the extracts enter interphase, and nuclei form when DNA or chromatin is added. We find that three pore proteins are present in a soluble form in this interphase extract. Two studies published during the course of these experiments found an overlapping set of equivalent Xenopus proteins present in an assembled form (Scheer et al., 1988; Featherstone et al., 1988). These proteins were found in the nuclear envelopes of Xenopus oocytes, which precede the formation of eggs in development. Thus, it is likely that the soluble pore proteins we observe derive from the disassembly of the oocyte nuclear envelope at meiosis and are then able to reassemble into nuclei during interphase. The molecular modification that triggers mitotic solubilization of the N-acetylglucosamine-bearing pore proteins and is reversed for pore assembly remains to be discovered.

Our observations demonstrate that the Xenopus WGA binding pore proteins are not bound to membrane vesicles. Based on the further findings that these proteins do not associate with chromatin in the absence of membranes, and that nuclear membranes and pores can form without these proteins, we conclude that these proteins do not play a critical recognition role in binding membrane vesicles to chromatin at the end of mitosis. The bulk of the nuclear envelope can assemble without an involvement of the WGA binding pore proteins. The assembly of partial pores (prepores) on the surface of chromatin was observed by Sheehan et al. (1988) when they used primarily the soluble fraction of a similar egg extract. From our results, one would predict that partial pores would also form in the soluble fraction of a depleted extract. Isolation of such intermediates will be valuable in defining steps in pore assembly.

Finally, we have developed conditions in which, by removal of a subset of nuclear pore proteins, we can create "biochemically mutant" pores. A normal pore has two very different modes of entry: passive diffusion and active transport. The biochemically altered pores formed here allow diffusion but lack active transport. Thus, the presence of the WGA binding proteins appears to be required to confer upon the channel the ability for a signal sequence–mediated expansion of the pore. By readdition of WGA binding pore proteins, we reconstitute functional pores. An immediate goal will be to reconstitute pores lacking single WGA binding pore proteins and, to this end, we are raising antibodies against individual pore proteins. It is hoped that this pore reconstitution system will allow not only an assignment of function to single WGA binding pore proteins, but that it will also aid in the identification of pore components essential to other pore structures and functions, such as the proteins that comprise the bulk of the pore and its channel and the proteins that control RNA export.

Experimental Procedures

Nuclear Reconstitution Extracts

Nuclear reconstitution extracts were prepared from Xenopus egg ex-

tracts as previously described (Newmeyer et al., 1986b; Newport, 1987). In this protocol, eggs were dejellied in 2% cysteine (pH 7.8), washed in lysis buffer (10 mM HEPES [pH 7.4], 250 mM sucrose, 50 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, 0.02 mg/ml cycloheximide, 0.005 mg/ml cytochalasin B, 10 μg/ml aprotinin, 10 μg/ml leupeptin), and packed with a low speed spin in a clinical centrifuge. The excess buffer was removed and the eggs were lysed by centrifugation at 10,000 rpm for 10 min in a Sorvall centrifuge. The lipids were discarded and the low speed supernatant (Newport, 1987) was removed from the pellet of yolk and pigment granules. The supernatant was centrifuged further in a Beckman TL-100 centrifuge (200,000 × g for 1 hr) to separate soluble and membrane components. Lipids that collected at the top were discarded, and the high speed soluble supernatant was set aside at 4°C to be used in transport assays or protein purification. This high speed soluble fraction of the extract could also be frozen at −70°C for some applications. One microliter of soluble supernatant was estimated to be derived from 1.6 Xenopus eggs. The membranes were collected and washed with 20 vol of Membrane Wash Buffer (MWB: 10 mM HEPES [pH 7.4], 250 mM sucrose, 50 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, 1 mM ATP, 5 μg/ml aprotinin, 5 μg/ml leupeptin), centrifuged in a Beckman TL-100 (15,000 rpm for 15 min), and resuspended in MWB to approximately 10× the original concentration present in the crude low speed supernatant. Sperm chromatin was prepared by demembranating sperm nuclei as described in Lohka and Masui (1983).

Preparation of Peptide-Protein Conjugates

Synthetic peptides containing the SV40 large T antigen wild-type nuclear signal sequence (Cys-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val) were synthesized by the University of California (San Diego) Peptide-Oligonucleotide Synthesis Facility and coupled to HSA (Calbiochem) using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS: Pierce; Goldfarb et al., 1986; Newmeyer and Forbes, 1988). Conjugates carrying ~20 signal sequences per HSA molecule were used and labeled with tetramethylrhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) before use (Newmeyer and Forbes, 1988).

Depletion of WGA Binding Proteins

The high speed soluble fraction of the extract was depleted of WGA binding proteins by two consecutive incubations with .5 vol of packed, washed WGA-Sepharose beads (E.Y. Laboratories, Inc.) for 45–60 min at 4°C with rocking. The Sepharose was pelleted briefly in an Eppendorf centrifuge, leaving a supernatant depleted of the WGA binding proteins ("depleted extract"). To control for nonspecific interaction with the Sepharose, identical incubations with WGA-Sepharose were performed with the addition of a high sugar buffer (HSB; 0.5 M N-acetylglucosamine [GlcNAc], 8 mM triacetylchitotriose [TCT], 20 mM HEPES [pH 7.0], 100 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 0.5 mM ATP, 5 μg/ml aprotinin, 5 μg/ml leupeptin) to final concentrations of 0.125 M GlcNAc and 2 mM TCT. The addition of sugar resulted in a .25 dilution of the extract that was later controlled for as described in "Depleted and Reconstituted Transport Assays" below.

Preparation of Xenopus and Rat WGA Binding Pore Proteins

Xenopus WGA binding pore proteins were isolated as follows: a given volume of the high speed soluble fraction of an extract was combined with .5 vol of packed, prewashed WGA-Sepharose, tumbled at 4°C for 1 hr, and centrifuged in an Eppendorf centrifuge for 15 s; the supernatant was discarded. The WGA-Sepharose was washed with 20 vol of Reconstitution Buffer (R Buffer: 20 mM HEPES [pH 7.0], 100 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 0.5 mM ATP, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and the WGA binding proteins were eluted by incubation with .25 of the original soluble fraction volume of HSB (20 min, 4°C). This resulted in an ~4-fold concentration of the eluted proteins. The WGA-Sepharose was removed by centrifugation, and the supernatant containing the WGA binding proteins was stored on ice until use.

To isolate the rat liver WGA binding pore proteins, rat liver nuclei were prepared as previously described (Newmeyer et al., 1986b; Newport and Spann, 1987). Nuclei ($6–8 \times 10^8$) were washed once in R Buffer, centrifuged 45 s in an Eppendorf centrifuge, and resuspended in 500 μl of R Buffer containing 2% Mega 10 detergent (Calbiochem). The nuclei were detergent-extracted at 4°C with tumbling for 30 min. The extracted nuclear pellet was removed by centrifugation in an Eppendorf centrifuge for 45 s and discarded. The detergent extract containing the WGA binding pore proteins was incubated with WGA-Sepharose, and the proteins were eluted as described above for the Xenopus extract. The WGA binding proteins prepared were only partially purified in most experiments; however, the presence or absence of other proteins had no effect on the results. As a control, an identical detergent extract was incubated with WGA-Sepharose in the presence of competing sugar (0.125 M GlcNAc, 2 mM TCT in R Buffer) or incubated with unconjugated Sepharose (O-beads control eluate). In these controls, it was reasoned that only nonspecific binding would occur. The control Sepharose was washed and eluted in a manner equivalent to the experimental extract.

Depleted and Reconstituted Transport Assays

For standard transport assays, 1 μl of demembranated sperm chromatin (20,000/μl) and 2 μl of membranes (washed and resuspended as described above) were added to 17 μl of the soluble fraction of an extract containing an ATP-regenerating system (final concentration = 1–2.75 mM ATP, 9 mM creatine phosphate, 100 U/μl creatine kinase). (For larger assays these components were increased proportionally.) The mixture was incubated 60–120 min to allow nuclear formation, as assayed by exclusion of 150 kd fluorescein-labeled (FITC) dextran (Newmeyer et al., 1986b). Fluorescently labeled transport proteins (final concentration ~0.01 mg/ml) were added and aliquots removed at various times from 5–60 min later for assay of transport with the fluorescence microscope.

Nuclear reconstitution and transport assays using extracts applied to WGA-Sepharose or control Sepharose were carried out in essentially the same manner. To test the ability of extracts depleted of WGA binding proteins to support formation of nuclei and subsequent transport, 12 μl of depleted extract containing the ATP-regenerating system (0.5 μl) was combined with 5 μl of HSB (bringing the sugar concentration and dilution equivalent to that of the plus sugar control), 1 μl of sperm chromatin (20,000/μl), and 2 μl of a 10× concentrated solution of washed membranes. Nuclear formation was assayed at ~90 min by testing for the ability to exclude 150 kd FITC-labeled dextran (~1 mg/ml; Sigma). Once nuclei were formed (~90–120 min), TRITC-ss-HSA (1.25–2.5 μg/ml) was added as above and transported assayed 5–180 min later with the fluorescence microscope or microfluorimeter. (During formation, the depleted nuclei did not reach a fully rounded state as quickly as the control nuclei, as might be expected for nuclei greatly reduced in transport. For this reason, the transport substrate was not added until both control and depleted nuclei showed obvious exclusion of the FITC-dextrans.)

As a control designed to take into account any possible nonspecific effects on transport from the application of extract to Sepharose, 17 μl of extract that had been applied to WGA-Sepharose in the presence of competing sugar was supplemented with an ATP-regenerating system and combined with 1 μl of sperm chromatin (20,000/μl) and 2 μl of a 10× concentrated solution of washed membranes. Nuclei were allowed to form, and transport was assayed as for the depleted extract. In other controls, 12 μl of extract that had been applied to unconjugated Sepharose was supplemented with an ATP-regenerating system, 5 μl of HSB, 1 μl of sperm chromatin (20,000/μl), and 2 μl of a 10× concentrated solution of washed membranes before assaying for nuclear formation and transport.

To assay the ability of WGA binding proteins, partially purified from either Xenopus egg extracts or rat liver nuclei as described above, to reconstitute the transport activity of nuclei when added to depleted extracts, 12 μl of depleted extract containing the ATP-regenerating system (0.5 μl) was combined with 5 μl of eluted Xenopus or rat proteins in HSB (see "Preparation of Rat and Xenopus WGA Binding Proteins"), 1 μl of sperm chromatin (20,000/μl), and 2 μl of a 10× concentrated solution of washed membranes. Transport was assayed with the fluorescence microscope as described. For larger extracts, components were proportionally increased. In most experiments, 5 μl of rat proteins was added; however, to determine the minimum estimate of rat proteins needed, lesser amounts were used (1–4 μl).

In experiments where ATP was depleted for visualization of the pore binding step, nuclei were allowed to form, and apyrase (grade VIII; Sigma), which hydrolyzes both ATP and ADP, was added 30 min before the addition of TRITC-labeled transport substrate (Newmeyer et al., 1986b). In the absence of ATP, the TRITC-ss-HSA substrate has been found to be arrested in a bound state at the pore, which can be visual-

ized as a fluorescent punctate stain of the nuclear rim with the fluorescence microscope (as observed here and in Newmeyer and Forbes, 1988) or as TRITC-ss-HSA-coated gold particles bound to individual pores with the electron microscope (Newmeyer and Forbes, 1988).

Determination of Nuclear-Associated Proteins

To determine the soluble Xenopus WGA binding proteins that become incorporated into nuclei upon nuclear reconstitution, sperm chromatin (1×10^7) was added to 1 ml of depleted or control extract, membranes, and ATP-regenerating system in the ratios given above. Nuclei were allowed to form for 120 min, diluted with 3 ml of MWB, and centrifuged through a 0.5 ml sucrose cushion (1.5 M sucrose, 12.5 mM KCl, 0.75 mM $MgCl_2$, 0.25 mM EGTA, 0.25 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin) at 25,000 rpm for 30 min in a Beckman 50.1 rotor. Reconstituted nuclei were washed twice with MWB with 5 min spins in the Eppendorf centrifuge. The washed nuclei were resuspended in 10 mM Tris (pH 7.4), 15 mM NaCl, 250 mM sucrose, 1 mM DTT, 3 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin plus 0.1 mg/ml DNAase (Worthington), incubated at room temperature for 20 min, and added to an equal amount of gel sample buffer (20% glycerol, 6% SDS, 0.8 M Tris [pH 8.8], 0.02% bromophenol blue, 10% 2-mercaptoethanol). Samples were boiled 3 min before electrophoresis on a 10% SDS–PAGE gel. Because of the fragility of the nuclei, the average yield of recovered nuclei was 5%–10%. The presence of associated WGA binding proteins was determined by transfer of the proteins to nitrocellulose and probing with ^{125}I-WGA.

Fluorescence Microfluorimetric Quantitation of Transport

For large scale quantitation of transport in normal and depleted nuclei, assay volumes were increased to 0.5 ml with 10^6 nuclei/ml. FITC-labeled ss-HSA was used as a transport probe to allow analysis by the flow microfluorimeter (Finlay et al., 1989; Hartl and Forbes, unpublished data). Nuclei were prepared for flow microfluorimetry using a method similar to Blow and Watson (1987) with modifications. Transport assays to be quantitated were diluted to 3 ml with Buffer A+ (80 mM KCL, 15 mM PIPES [pH 7.5], 15 mM NaCl, 5 mM EDTA, 1 mM DTT, 2 mM $MgCl_2$) and fixed by the addition of 300 μl of 100 mM ethylene glycol bis-(succinimidylsuccinate)(EGS; Pierce) in dimethyl sulfoxide. The samples were incubated at room temperature for 10 min before the addition of 80 μl of 1 M Tris–HCL (pH 7.5) to stop the reaction. The samples were underlain with 15% and 70% sucrose cushions and centrifuged at 6800 rpm for 10 min in a Beckman SW 50.1 rotor. The nuclei, which sediment to the 15%–70% interface, were removed and added to an equal volume of 50 μg/ml RNAase and 50 μg/ml propidium iodide in Buffer A+. They were then incubated for 1 hr at room temperature, pelleted ~45 s in an Eppendorf centrifuge, and resuspended to a final concentration of 10^6 nuclei/ml in PBS plus 0.5 mM spermidine.3HCL and 0.15 mM spermine.4HCL. The resuspended nuclei were analyzed using a flow microfluorimeter (Flow Cytometry Lab, The Salk Institute), and the amount of fluorescence (FITC-ss-HSA) accumulated per individual nucleus was measured in relative fluorescence units.

Electron Microscopy

Samples were fixed, stained, and sectioned, and electron microscopy was performed as in Sheehan et al. (1988), except that the samples were embedded in Epon (Electron Microscopy Sciences, Fort Washington, PA), sectioned, and poststained with uranyl acetate and lead citrate. In some experiments, nuclei were diluted 5:1 with identical fixative, incubated overnight at 4°C, and concentrated by centrifugation at 1000 × g for 5 min. The pellet was washed and processed as above. Samples were examined using a Philips EM 300 electron microscope at 80 kV with a 50 mm objective aperture.

Gel Electrophoresis, Immunoblotting, and Radiolabeled Lectin Blotting

The proteins present in the nuclear or extract samples were prepared for gel electrophoresis on 10% SDS–PAGE gels and transferred to nitrocellulose as described in Finlay et al. (1987). Radiolabeling of WGA and probing of protein blots with radiolabeled ^{125}I-WGA was also performed as in Finlay et al. (1987). Immunoblotting with the anti-pore monoclonal antibody 414 (kindly provided by L. Davis, T. Meier, and G. Blobel; Davis and Blobel, 1986, 1987) was performed by incubation of a blot with 5% nonfat dry milk, 0.1% Tween 20 in PBS for 1 hr at ambient temperature. The blot was then incubated for 2 hr with 15 ml of a 1:800 dilution of MAb 414 (21 mg/ml) in 5% nonfat dry milk, 0.1% Tween 20 in PBS. The blot was washed three times in the same buffer with no antiserum and twice with PBS alone, incubated for 2 hr with ^{125}I-labeled protein A (ICN; 0.033 mg/ml) at a concentration of $2–4 \times 10^6$ cpm/15 ml, washed, and exposed for autoradiography.

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