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Permeability measurements with closed vesicles from rat liver nuclear envelopes

[protein transport/poly(A)-stimulated ATPase/mRNA efflux]

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ABSTRACT **Closed nuclear envelope ghosts in the physiological orientation were prepared from rat liver nuclei as previously described. Here we report transport measurements of various proteins and ribonucleic acids across the envelope of these vesicles. Histones were accumulated rapidly in the ghosts, in contrast to other, nonnuclear, proteins. Triton X-100 removal of the external nuclear membrane from loaded vesicles, as well as comparative studies with open vesicles, excluded the effects of external adsorption. The exchange rate of histones across the nuclear envelope is strongly depressed in the presence of GTP and GDP. The vesicles contain the translocation mechanism for poly(A)-containing RNA. The translocation of poly(A), messenger RNA, and ribosomal RNA was investigated after entrapment of these nucleic acids during the preparation of vesicles. Our data show that the complete export of only poly(A)-containing RNA from the vesicles is enhanced in the presence of 2 mM ATP. This RNA, as well as poly(A), is transported unidirectionally.**

Previously, we described the preparation and characterization of closed envelopes from rat liver nuclei (1, 2). These vesicles were shown to be tight. The DNA content, calculated per mg of protein, is reduced to 1–1.5% of the value for whole nuclei.

The high number of binding sites for nuclear constituents, such as histones or mRNA, renders the exact measurement of rates of migration into or out of whole nuclei difficult. Evidence from microinjection experiments (3, 4), sequence analysis, and especially site-directed mutagenesis of proteins that accumulate in the nucleus (5, 6) has prompted the discussion of selectivity of nuclear membrane transport (7). The nuclear envelope ghosts seemed suitable for the further elucidation of these mechanisms.

MATERIALS AND METHODS

Leaky nuclear envelopes ("DNase envelopes") were prepared by hypotonic DNase I treatment as described by Dwyer and Blobel (8). The preparation of closed nuclear envelopes with heparin solutions was carried out as previously described (1, 2).

Poly(adenylic acid) of an average molecular weight of 100,000 was purchased from Sigma, and the absence of poly(A) with molecular weight below 60,000 was verified by HPLC runs on a Nucleogen DEAE 500-10 column. NTPase activity was determined as described (1). Histones were prepared from calf thymus and fractionated by the procedure of Johns (9) (purity control, see Fig. 3 and ref. 10). *Xenopus laevis* eggs were obtained from the Max-Planck-Institute of Biophysics, Frankfurt, and nucleoplasmin was prepared from these as described (11). All proteins were labeled in 50 mM $NaHCO_3$ by addition of [^{14}C]acetic anhydride (Amersham Buchler), specific activity 120 mCi/mmol (1 Ci = 37 GBq). The proteins were then dialyzed against the buffer described in the legend of Fig. 3. Their final specific activity amounted to 30,000–50,000 cpm/mg. Thus, between 1 and 2 side chains per 15 kDa of protein were modified. Fluorescence labeling of histones was achieved by incubation of the protein at 5 mg/ml in 0.01% $NaHCO_3$ solution with fluorescein isothiocyanate for 4 hr and thorough dialysis. The molar ratio was adjusted to furnish a modification of 3.5 amino acid side chains per protein molecule.

mRNA and rRNA were prepared from rat liver polysomes (12–14), and iodinated with ^{131}I according to Commerford (15). Poly(A) was labeled with [^{32}P]phosphate in the presence of polynucleotide kinase (BioLabs) and the enzymes generating [γ-^{32}P]ATP from glyceraldehyde 3-phosphate, ADP, and inorganic phosphate (16, 17).

RESULTS

Morphology. It had already been shown by Bornens and Courvalin (18) that heparin extraction of chromatin from nuclei leaves their pore complexes intact, as characterized by electron microscopy. As shown in Fig. 1, the closed vesicles still possess residual fringes of the chromatin material on the inside of the inner membrane; the pore complexes are revealed by freeze fracture. Phase-contrast light microscopy reveals a population of single particles. We measured 250 randomly selected vesicles; among these, 73% had diameters between 6.0 and 8.5 μm, with a range between 5 and 10.2 μm. Freshly prepared DNase envelopes (see *Materials and Methods*) have a very similar appearance in the light microscope (Fig. 1*A Inset*). However, they do not carry out the transport phenomena described below.

Measurement of Permeability. The method developed by Klingenberg and Pfaff (19) for transport measurements in mitochondria was slightly modified. In Microfuge (Beckman) tubes, vesicles from 0.15 ml of a suspension at a protein concentration of 2 mg/ml were rapidly centrifuged through a layer of 0.04 ml of silicone oil into 0.01 ml of 60% $HClO_4$ solution to form a tight pellet (2).

To determine the yield and efficiency of the method, envelopes labeled with acetic anhydride were used. Fig. 2*A* shows that, up to a protein concentration of 5 mg/ml, the loss of labeled material during the separation remained negligible. An example of a typical histone uptake measurement is shown in Fig. 2*B*. The total amount of protein in pellets and supernatants always amounted to at least 95% of the added radioactivity.

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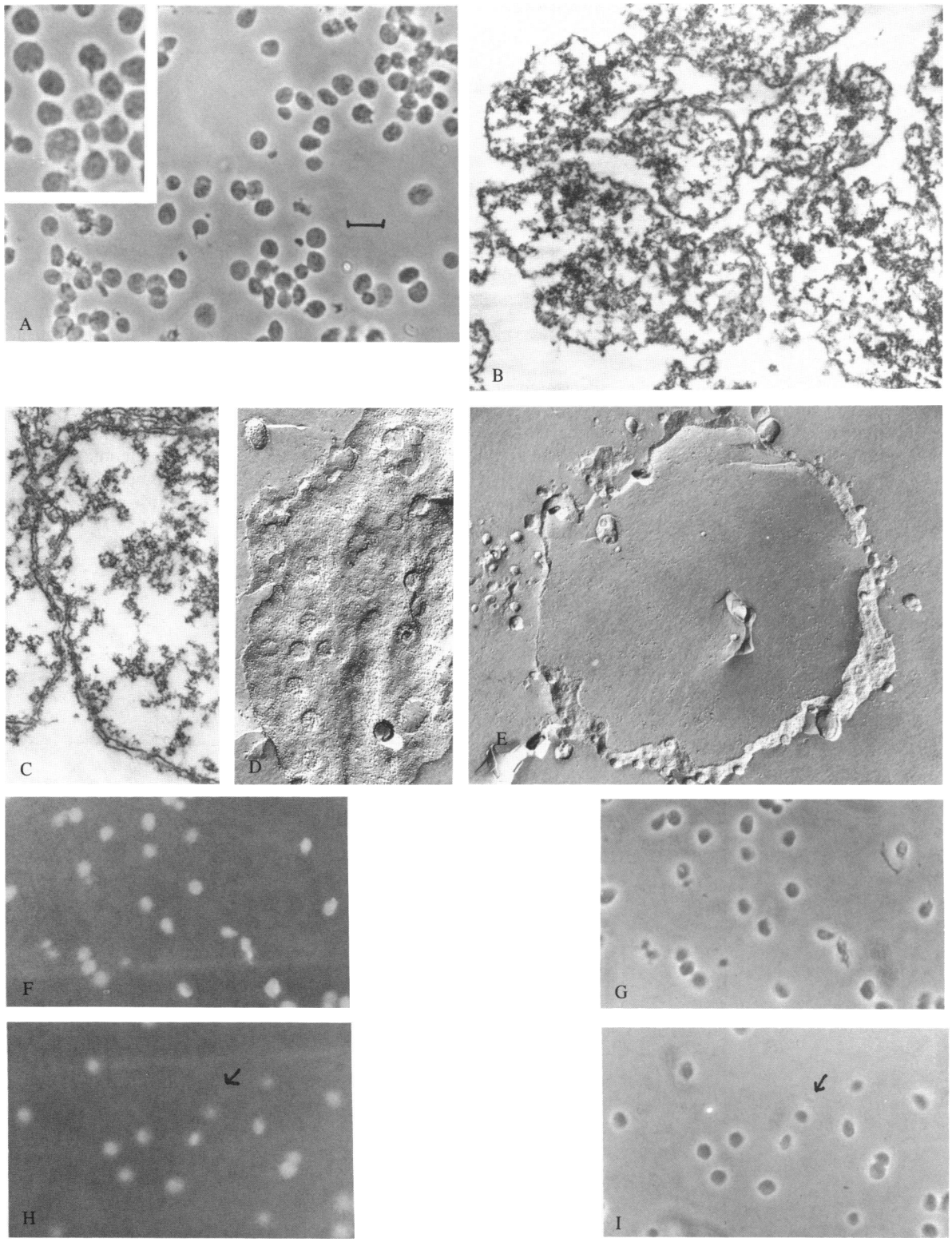

FIG. 1. (*A*) Light micrograph of closed heparin vesicles. (Bar = 10 μm.) (*Inset*) Light micrograph of DNase envelopes. (×660.) (*B* and *C*) Electron micrographs of thin sections of heparin vesicles, fixed with 2.5% (wt/vol) glutaraldehyde in 50 mM Tris·HCl, pH 7.4/5 mM KCl/5 mM $MgCl_2$/3.3 mM $CaCl_2$/0.25 M sucrose buffer for 30 min and postfixed with 1% OsO_4 solution for 1 hr. (×7600 and ×19,000, respectively.) (*D* and *E*) Electron micrographs of replicas of freeze-etched fixed vesicles, freeze fracturated at −130°C in a Balzers Fa 300. (*D*) Pore complexes on a tangential fracture face (×38,000.) (*E*) Cross-fractured intact nuclear envelope (×22,000.) (*F–I*) Ubiquitous distribution of accumulated fluorescence-labeled histones in the vesicles. The accumulation was allowed to proceed under the conditions described in the legend to Fig. 3 *A* and *B*, but in the presence of 10 mM GTP for 20 min. The suspension was then cooled to 0°C, and the vesicles were washed twice with the same buffer by centrifugation at 3000 × *g*. After resuspension, the vesicles were photographed as they floated in a drop of the buffer under illumination with a UV source above (*F* and *H*) or with a visible light source below (*G* and *I*). (×600.) (*F* and *G*) Freshly prepared vesicles, same field. (*H* and *I*) Vesicles treated with 1% Triton X-100 after accumulation of fluorescence-labeled histones, as described in the text; same field.

Accumulation and Exchange Rates for Proteins. A mixture of histones is accumulated within the envelopes much faster than cytochrome *c*, an even smaller basic protein (Fig. 3*A*), whereas the large nonnuclear protein IgG is evidently com-

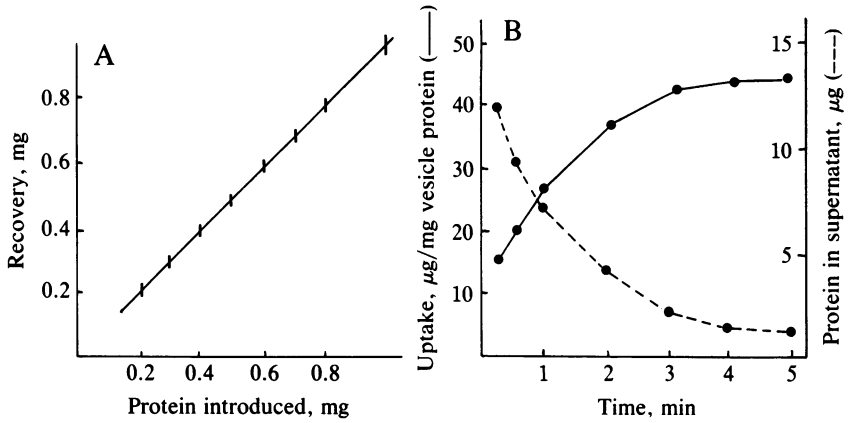

FIG. 2. (*A*) Increasing amounts of labeled nuclear envelopes in the buffer defined in the legend to Fig. 3 were subjected to centrifugation through a silicone oil layer, as described in the text, and the yield in the pellet fraction was determined. (*B*) Uptake of a mixture of histones, as described in Fig. 3. ——, Increase of labeled protein in the pellet fraction; ---, decrease of the labeled protein in the supernatant.

pletely excluded. The same figure shows the adsorption of histones to the leaky DNase envelopes. This adsorption value is much lower than the accumulation value of the closed vesicles, although the DNA content of DNase envelopes (2.5–3% of the original content in the whole nucleus, calculated per mg of protein) is higher in this case. The yield of the centrifugation method was also monitored in the case of the DNase envelopes, as demonstrated in Fig. 2, with the same result. Polylysine uptake, not unexpectedly, follows the histone curve, as well as that of nucleoplasmin.

The accumulation of histones, nucleoplasmin, and polylysine, in contrast to that of the nonnuclear proteins tested, is further accelerated by more than 30% of the initial rate in the presence of 5 mM GTP (Fig. 3*B*) or GDP, but not of GMP,

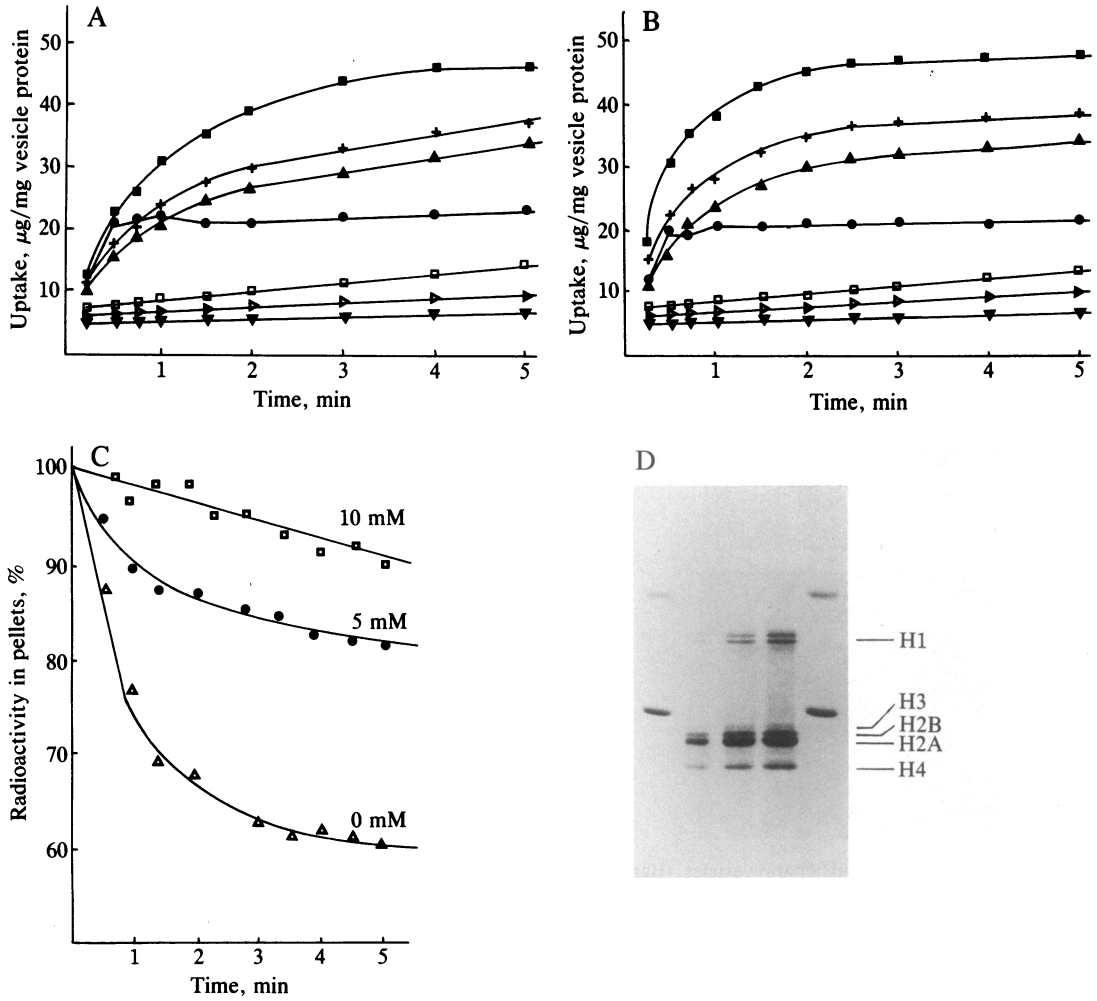

FIG. 3. (*A* and *B*) Freshly prepared envelopes were suspended in 50 mM Tris·HCl buffer, pH 7.4, containing 25 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM NaCl, 2.5 mM Na_2HPO_4, and 5 mM spermidine, to a protein concentration of 2 mg/ml at 22°C. Samples, 0.15 ml each, were layered upon a silicone oil cushion, and the chosen labeled protein was added to a final concentration of 100 μg/ml at zero time. Only the increase in the pellet fraction is shown here. ■, Mixture of histones; ▲, polylysine; ●, mixture of histones and DNase envelopes; ▷, cytochrome *c*; ▼, IgG; +, nucleoplasmin from *Xenopus laevis*; □, myogloglobin. (*A*) No other additions; (*B*) in the presence of 5 mM GTP. (*C*) Measurement of the exchange rate of internal radioactively labeled histones with a 10-fold excess of external unlabeled histones, in the presence of GTP as indicated. (*D*) $NaDodSO_4$/PAGE of the purified histone fractions used.

adenine, or uracil nucleotides. With DNase envelopes, this GTP/GDP effect was not observed.

This observation led us to study the exchange rate of histones in the presence and absence of GTP. The vesicles were incubated with labeled histones under the conditions described in the legend of Fig. 3 for 10 min. After a 10-min centrifugation at 4500 × *g*, the pellet was resuspended in the original volume of buffer, containing a 10-fold excess of unlabeled histones. The decrease of labeled histones within the envelopes and their appearance in the extravesicular volume was measured as described above (Fig. 3*C*).

Since in previous experiments the internal volume of the envelopes had been determined to be 8.6 μl/mg of vesicle protein (2), 0.0026 ml of the total of 0.15 ml of envelope suspension corresponds to the internal compartment under our conditions. Thus the uptake of histones, as shown in Fig. 3*A*, represents a 25-fold accumulation, when compared with the equilibrium value.

External Adsorption, Controls. The possibility that the observed uptake kinetics were due to the external adsorption of histones onto the closed vesicles, thus yielding an artificially high level of bound histone, had to be excluded. The comparison of the closed envelope ghosts with the leaky DNase envelopes, and the lack of the GTP effect, as shown in Fig. 3 *A* and *B*, served as one control.

The treatment of nuclear envelopes with 1% Triton under controlled conditions (20) will remove the outer, and parts of the inner, nuclear membrane (21, 22) but leaves the lamina rim intact. In our experiments, closed envelopes were loaded with labeled histones for 30 min in the presence of 5 mM GTP, as described in the legend of Fig. 3. The suspension was then cooled to 4°C and washed twice by centrifugation in the same buffer at 4°C, and then buffered Triton X-100 solution was added to a final concentration of 1%. After 10 min at 4°C, the vesicles were again washed by centrifugation in cold buffer, containing 5 mM GTP, three times. With all proteins tested in Fig. 3, the value of bound protein in the pellet fraction was reduced by 10–20% after Triton X-100 treatment.

The uniform function of the vesicles was demonstrated by the uptake of fluorescence-labeled histones in the presence of GTP, as described in the legend of Fig. 1 *F–I*. A few of the vesicles show a reduced fluorescence (Fig. 1 *F* and *G*), as do the leaky DNase-prepared vesicles (not shown). This effect becomes much more distinct when the vesicles are washed with Triton X-100 solution as described above. The few leaky vesicles then show only a very weak, ring-like fluorescence (arrow, Fig. 1 *H* and *I*). Their amount varied between 5% and 12% in our preparations.

When the $HClO_4$ solution layer was replaced by an equal amount of buffered 20% sucrose solution during the measurement of uptake of fluorescence-labeled histones, light and electron microscopy, as described in the legend of Fig. 1, demonstrated the intact structure of the envelopes.

Finally, adsorbed heparin might have caused heightened adsorption of histones. The extraction procedure was therefore repeated with radioactively labeled heparin. It was found that heparin is completely removed from the vesicle preparation during extraction and washings.

Tightness of Closed Envelopes. In our previous experiments, the evidence for the tightness of the heparin-extracted envelopes was drawn from the inclusion of ferritin and the exclusion of *S*-dinitrophenyl-6-mercaptopurine riboside triphosphate. Its inhibitory effect upon the NTPase activity of the nuclear envelope cannot be observed with whole nuclei, but it is distinct after disruption of the envelope. The same effect was observed by comparison of the leaky DNase envelopes with the closed heparin vesicles (1).

The permeability of the closed envelopes for poly(A) of an average molecular weight of 100,000 was tested under the conditions given in the legend of Fig. 3. Only a small fraction was bound to the pellets (Table 1), and this fraction remained constant during a 60-min incubation. This amount would correspond to only 10–15% of the internal volume of the vesicles; therefore, poly(A) is to a large part excluded by the closed envelopes. The same result was found for poly(A)-containing mRNA. It also indicates that up to 15% of the envelopes are leaky, in accordance with previous experiments (1).

Table 1. Uptake of RNA fractions by closed heparin vesicles

Preparation	RNA	Conc., μg/ml	Uptake, ng RNA/mg vesicle protein
Heparin envelopes	Poly(A)	10	2
	Poly(A)	50	12
	Poly(A)	100	27
	mRNA	50	13
	mRNA	100	22
DNase envelopes	Poly(A)	100	25
	mRNA	50	10

^{131}I-labeled mRNA and ^{32}P-labeled poly(A) were employed at the final concentrations indicated, and under the experimental conditions given in the legend to Fig. 3 and the text.

Envelope NTPase activity is stimulated by ribonucleic acids, especially by poly(A) (23). As shown in Table 2, this effect is distinct in the leaky DNase envelopes. Closed envelopes, however, show it only to a small extent when poly(A) or rat liver mRNA is offered after resealing. When poly(A) is introduced into the vesicles during their preparation, activation of the nuclear envelope NTPase reaches the same level as in the DNase envelopes. From these experiments it may be deduced that up to 85–90% of the vesicles are tight.

Efflux of RNA from Vesicles. During the heparin extraction step, vesicles were loaded with either poly(A)-containing RNA prepared from rat liver polysomes or rat liver ribosomal RNA. The vesicles were washed twice by centrifugation at −3°C in the buffer indicated in the legend to Fig. 4. The final concentration of RNA in the vesicle preparation then amounted to 7.2 ng/mg of protein. After resuspension at a protein concentration of 2 mg/ml, efflux measurements were carried out as described above for the uptake of histones. While the efflux of ribosomal RNA was not influenced by ATP, the efflux of poly(A)-containing mRNA was distinctly enhanced in the presence of 2 mM ATP, as shown in Fig. 4. The efflux reached a plateau at approximately 20 min, at which time >90% of all poly(A) and mRNA contained within the vesicles was exported, indicating that the process did not reflect a preferential efflux of small or partially degraded poly(A)-mRNA species. To test this further, the mRNA was

Table 2. Activation of internal NTPase by poly(A) and mRNA

Preparation	RNA addition from outside: RNA	RNA addition from outside: Conc., μg/ml	Poly(A) inclusion, ng/ml internal volume	NTPase, nmol P_i/mg protein per min
Heparin envelopes	None		—	81
	Poly(A)	10	—	85
	Poly(A)	50	—	91
	Poly(A)	100	—	92
	mRNA	50	—	91
	mRNA	100	—	91
	—		5	107
	—		8	129
DNase envelopes	None		—	49
	Poly(A)	60	—	76
	Poly(A)	100	—	92

Internal NTPase activity was measured as described in ref. 1.

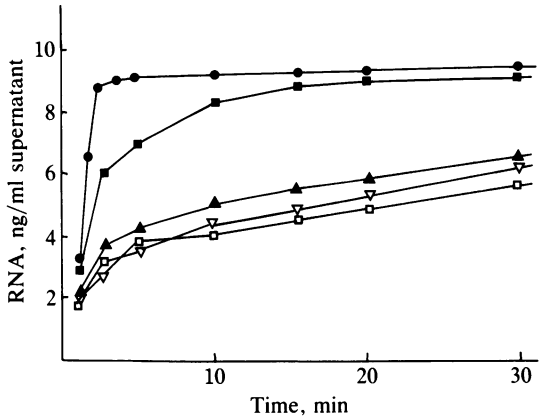

FIG. 4. Ribonucleic acid efflux. Vesicles were loaded in the heparin extraction step with ^{131}I-labeled nucleic acid at 40 μg/ml and cooled to −3°C. After centrifugation at this temperature for 10 min at 4500 × *g*, the vesicles were resuspended in a 10 mM Tris·HCl buffer, pH 8.0, containing 100 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, and 0.5 mM $CaCl_2$ at −3°C to a final protein concentration of 1 mg/ml and washed twice in this medium by centrifugation. Efflux was measured after centrifugation of aliquots through silicone oil layers, as described in *Materials and Methods*, but in this case 130-μl samples of the supernatants in the Microfuge tubes from the various incubation times were withdrawn for scintillation counting. ▲, mRNA in the absence of ATP; ■, mRNA in the presence of 2 mM ATP; ●, poly(A) in the absence of ATP; ▽, ribosomal RNA in the absence of ATP; □, ribosomal RNA in the presence of 2 mM ATP.

subjected to agarose gel electrophoresis before and after the efflux experiments. Supernatants were collected 30 min after the ATP pulse, precipitated with ethanol, and redissolved in transport buffer. Equal amounts of radioactivity before and after the efflux measurements were applied to the gel, autoradiographs then demonstrated that the RNA had not been degraded during this process.

DISCUSSION

Previous experiments (24, 25) led to the conclusion that pore complexes permit macromolecules to enter the nucleus up to a molecular weight of approximately 40,000. On the other hand, our experiments show that substrates such as poly(A) or poly(A)-mRNA cannot penetrate the envelope from the outside, but will quickly be exported, especially upon an ATP signal.

The fast initial uptake of histones and the influence of guanosine nucleotides also point to a selection mechanism at the level of the nuclear envelope. This is further suggested by the presence of a distinct signal sequence (6) responsible for an accumulation in the nucleus. Although histones are bound to associated proteins in the living cell (11), it seems reasonable to presume that such characteristic structural details are sufficiently accessible for an interaction with the pore complex. The same reasoning holds for the poly(A) sequences ofmRNA–protein particles. At least a part of the poly(A) is amenable to binding by poly(dT) (26).

From these experiments it may be concluded that the polyadenylylation of mRNA in eukaryotes constitutes one decisive signal for export from the nucleus. When the poly(A) tail is removed from mRNA by digestion with endonuclease IV (27), the export of the nucleic acid from the vesicles described here is distinctly reduced, as demonstrated previously by P. Agutter (personal communication). We have confirmed this experiment.

It is our current working hypothesis, therefore, that essential features of proteins accumulated in the nucleus, as well as of nucleic acids to be exported, are free to interact with components of the nuclear envelope, in all probability with the pore complex.

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