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Differential Effect of Insulin and Epidermal Growth Factor on the mRNA Translocation System and Transport of Specific Poly(A+) mRNA and Poly(A-) mRNA in Isolated Nuclei†

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Received May 31, 1989; Revised Manuscript Received October 6, 1989

ABSTRACT: The efficiency of efflux of rapidly labeled poly(A)-containing mRNA from isolated rat liver nuclei was found to be modulated by insulin and epidermal growth factor (EGF) in a biphasic but opposite way. At physiological concentrations (10 pM insulin and 1 pM EGF), maximal stimulation of the transport rate by insulin (to 137%) and maximal inhibition by EGF (to 69%) were obtained; at higher concentrations (>100 pM and >10 pM, respectively), the amount of poly(A)-containing mRNA released into the postnuclear supernatant was nearly identical with the level found in untreated nuclei (=100%). Using mRNA entrapped into closed nuclear envelope (NE) vesicles as a model system, it was found that the modulation of nuclear efflux of mRNA by the two growth factors occurs at the level of translocation through the nuclear pore. The NE nucleoside-triphosphatase (NTPase) activity, which is thought to mediate nucleocytoplasmic transport of at least some mRNAs, responded to insulin and EGF in the same manner as the mRNA transport rate. The increase in NTPase activity caused by insulin and the decrease in NTPase activity caused by EGF were found to be due to changes of the maximal catalytic rate; the Michaelis constant of the enzyme remained almost constant. Investigating the effect of the two growth factors on transport of specific mRNAs, poly(A)-containing actin mRNA was found to display the same alteration in efflux rate as rapidly labeled, total poly(A)-containing mRNA. In contrast, efflux of histone H4 mRNA, which lacks a 3'-poly(A) sequence, decreased in response to insulin and reached minimum levels at the same concentration at which maximum levels of actin mRNA transport rate were obtained. Studying the mechanism of action of insulin and EGF on NE mRNA translocation system, insulin was found to cause an enhancement of NE-associated phosphoprotein phosphatase activity, resulting in a dephosphorylation of the NE poly(A) binding site (=mRNA carrier) and, hence, in a decrease in its affinity to poly(A) [the poly(A) binding affinity of the poly(A)-recognizing mRNA carrier within the envelope is increased after phosphorylation]. EGF, on the other hand, stimulated the protein kinase, which phosphorylates the carrier, and, hence increased the NE poly(A) binding affinity. Because the stage of phosphorylation of the mRNA carrier (which is coupled with the NTPase within the intact NE structure) is inversely correlated with the activity of the NTPase, an enhancement of poly(A)-containing mRNA transport rate by insulin and an inhibition by EGF are observed.

Transport of mRNA from the nucleus to the cytoplasm across the nuclear envelope (NE)[1] can be subdivided into three steps (Schröder et al., 1987a; Agutter, 1988): (1) release of mRNA from the intranuclear binding site (nuclear matrix; Schröder et al., 1987b); (2) translocation of the mRNA

† This work was supported by Grants Schr 277/2-1 and Mu 348/7-7 from the Deutsche Forschungsgemeinschaft.
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[1] Abbreviations: EGF, epidermal growth factor; NE, nuclear envelope; NTPase, nucleoside-triphosphatase; PK, protein kinase; poly(A+) mRNA, poly(A)-containing mRNA; poly(A-) mRNA, poly(A)-free mRNA; PPH, phosphoprotein phosphatase; $PhMeSO_2F$, phenylmethanesulfonyl fluoride; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/90/0429-2368$02.50/0

through a nuclear pore (Agutter et al., 1976; Skoglund et al., 1983); and (3) binding of the transported mRNA to the cytoskeleton. While step 1 (release from nuclear matrix) seems to be responsible for the selectivity of transport for mature mRNA species (immature mRNAs are normally restricted to the nucleus; Schröder et al., 1987b), increasing evidence is available that step 2 (pore passage) is involved in regulating the quantity of the mRNA exported out of the nucleus (Schröder et al., 1986c, 1988a,b, 1989a). The underlying mechanism is assumed to be a phosphorylation/dephosphorylation-related change in affinity of the NE mRNA binding site (=mRNA carrier; McDonald & Agutter, 1980; Schröder et al., 1988b) to the poly(A) sequence of mRNA; this mechanism can be modulated by cytosolic mRNA transport stimulatory proteins (Moffett & Webb, 1983; Schröder et al., 1986c). An enhanced phosphorylation of the NE, mediated by both endogenous NII-like protein kinase (PK-NII; Schröder et al., 1988b) and protein kinase C (PK-C; Rottmann et al., 1987; Schröder et al., 1988a,b), has been shown to be linked with a reduced NTPase activity (Schröder et al., 1986c), resulting in a decreased mRNA transport rate (Schröder et al., 1988a,b). The latter enzyme, which has been purified by us to homogeneity (Schröder et al., 1986b), is assumed to provide the energy for nuclear export of poly(A)-containing mRNA [poly(A+) mRNA]; this process has been shown to require hydrolysis of ATP or GTP (Agutter et al., 1976; Schröder et al., 1989a). On the other hand, transport of poly(A)-free mRNA [poly(A−) mRNA; e.g., replication-dependent histone mRNA] does not depend on cleavage of high-energy phosphodiester bonds, but it is promoted in the presence of nucleotides or nonhydrolyzable nucleotide analogues (Schröder et al., 1989a).

The NE has been shown to contain specific receptors for hormones [e.g., insulin (Goldfine & Smith, 1976; Vignery et al., 1978; Goidl, 1979) and triiodothyronine (Goldfine & Smith, 1976)], growth factors [e.g., epidermal growth factor (EGF; Johnson et al., 1980; Murthy et al., 1986) and nerve growth factor (Yankner & Shooter, 1979)], and other effectors [e.g., tryptophan (Schröder et al., 1989b)]. Purrello et al. (1982, 1983) have demonstrated that binding of insulin to its NE receptor results in an increased activity of NE NTPase, resulting in an enhanced mRNA efflux from isolated nuclei. This effect was assumed to be due to a dephosphorylation of the NTPase, caused by the hormone (Purrello et al., 1983). Later results, however, showed that the NTPase is not a phosphoprotein (Clawson et al., 1984; Schröder et al., 1986b) and its modulation by phosphorylation is caused by interaction of the enzyme with the mRNA carrier within the envelope (Bachmann et al., 1984; Schröder et al., 1986c, 1987a, 1988b) and possibly other NE components (Schröder et al., 1988a). Studies on nuclear transport using the fluorescence photobleaching technique revealed that, besides insulin, also other molecules such as EGF and wheat germ agglutinin are able to modulate exchange of macromolecules across the NE (Schindler & Jiang, 1987); this has been attributed to an interaction of these effectors with an actomyosin-like contractile apparatus within the pore structure (Schindler & Jiang, 1986). However, the concentrations applied in these experiments were unphysiologically high.

In the present study, we demonstrate that transport of mRNA does not uniformly respond to two modulators of nucleocytoplasmic exchange processes (EGF and insulin): While the nuclear export of poly(A+) mRNA (actin or total adenylated mRNA), which depends on ATP hydrolysis, was enhanced by insulin, energy-independent efflux of poly(A−) mRNA (histone H4) was decreased. EGF, on the other hand, inhibited the transport of adenylated messengers, while poly(A−) mRNA export was unaltered. The response of both mRNA transport processes to insulin and EGF was found to be biphasic, paralleling the changes in NE NTPase activity. Evidence is presented that both growth factors affect down-regulation of the NTPase by the phosphorylated mRNA carrier protein p106 (Schröder et al., 1988b) within the envelope, most likely by stimulating either NE PK (EGF) or NE protein phosphatase (PPH) activity (insulin), resulting in an alteration in the stage of phosphorylation of the mRNA carrier (Schröder et al., 1986c, 1987a) and, hence, in a change in the poly(A+) mRNA transport rate.

Materials and Methods

Materials. The following materials were obtained: [γ-^{32}P]ATP (specific activity, 3000 Ci/mmol), [γ-^{32}P]GTP (10 Ci/mmol), [α-^{32}P]dTTP (3000 Ci/mmol), [5,6-^{3}H]uridine (50 Ci/mmol), [^{3}H]ATP (11 Ci/mmol), [^{3}H]GTP (17 Ci/mmol), and [^{3}H]poly(A) (600 Ci/mmol of nucleoside residue) from Amersham Buchler International (Buckinghamshire, England); 8-azido[α-^{32}P]ATP (specific activity, 6.3 Ci/mmol) from ICN (Eschwege, FRG); DNase I (bovine pancreas; LSOO 02172) from Worthington (Freehold, NJ); poly(A), poly(C), DNA polymerase I (*Escherichia coli*, 11388 units/mg), RNase A (bovine pancreas, 50 Kunitz units/mg), RNasin (RNase inhibitor), phospho*enol*pyruvate, and pyruvate kinase from Boehringer Mannheim (Mannheim, FRG); insulin (bovine pancreas; 24 IU/mg; I6634), epidermal growth factor (EGF; mouse submaxillary gland; E1257), creatine phosphokinase, phosphocreatine, spermidine, phenylmethanesulfonyl fluoride ($PhMeSO_2F$), β,γ-methylene-ATP (purified by high-performance liquid chromatography), ribonucleoside–vanadyl complexes, 1,2-dioleyl-*rac*-glycerol, histone III-S, *O*-phospho-DL-serine, and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) from Sigma (St. Louis, MO); yeast RNA (s_{20} = 8 S; RNA content higher than 97%) from Serva (Heidelberg, FRG); and messenger-activated paper (mAP) from Medac (Hamburg, FRG). Staurosporine, an inhibitor of PK-C (Tamaoki et al., 1986), was a gift of Kyowa Medex Co. (Tokyo, Japan).

Preparation of Nuclei and Nuclear Substructures. Nuclei were isolated from rat liver (2–3-month-old male Wistar rats) according to Blobel and Potter (1966), except that 1 mM $PhMeSO_2F$ and 5 mM 2-mercaptoethanol were added to all the buffers used. Nuclear matrices were prepared by the method of Comerford et al. (1986) and were stored at −20 °C in 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, 250 mM sucrose, and 1 mM $PhMeSO_2F$. Nuclear envelopes were isolated as described by Kaufmann et al. (1983) and were stored at −70 °C in 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 2.1 M sucrose, and 1 mM $PhMeSO_2F$. Pore complex laminae were prepared from the envelopes as described by Aaronson and Blobel (1975). If indicated, nuclei were freed from nuclear membranes by treatment with 1% Triton X-100 in ATP-free transport medium (see below) for 10 min at 0 °C (Schröder et al., 1988b). The absence of the outer nuclear membrane or both membranes was assessed electron microscopically as described previously (Schröder et al., 1988b).

In some experiments, prelabeled nuclei or nuclear matrices were used, which were obtained from rats after intraperitoneal injection of [5,6-^{3}H]uridine (5 μCi/g body weight) 30 min before they were killed.

Enzyme Preparations. The nuclear envelope NTPase was purified to apparent homogeneity from rat liver envelopes by

the method of Schröder et al. (1986b).

Nuclear protein kinases NI and NII were prepared from rat liver; both enzyme activities were separated by passage through a DEAE-Sepharose column (Yutani et al., 1982). Final purification was achieved by affinity chromatography on Sepharose 4B–sebacic acid hydrazide–β,γ-methylene-ATP (Schröder et al., 1988b).

Protein kinase C was purified from rat brain by binding to human erythrocyte inside-out vesicles and phenyl-Sepharose chromatography, essentially as described by Wolf et al. (1985).

Total cytosol protein was obtained from the 100000*g* supernatant of a rat liver homogenate. The supernatant was depleted of mRNA efflux stimulatory proteins by streptomycin sulfate and ammonium sulfate precipitation (Moffett & Webb, 1983), followed by dialysis against 50 mM Tris-HCl buffer (pH 7.6, containing 2.5 mM $MgCl_2$/25 mM KCl).

Preparation of Tritium-Labeled Poly(A+) mRNA and Poly(A−) mRNA. Polysomes were prepared from the livers of male Wistar rats (12–24-months old) 60 min after intraperitonal injection with [5,6-^{3}H]uridine (1 μCi/g body weight) (Schröder et al., 1986c). Poly(A+) mRNA was isolated from the polysomes by phenol/chloroform extraction and oligo(dT)–cellulose chromatography (Maniatis et al., 1982). Poly(A−) mRNA was obtained by digestion of the poly(A+) mRNA with purified endoribonuclease IV (Müller, 1976), as described previously (Bernd et al., 1982a).

In Vitro Systems for Measuring RNA Release and Efflux. Release of RNA from nuclear matrices was determined by incubating the matrices (24 μg of protein/assay) in a final volume of 100 μL with 50 mM Tris-HCl (pH 7.5), 10 μM ATP, 1 mM $MgCl_2$, 100 mM KCl, 250 mM sucrose, and 1 mM $PhMeSO_2F$ at 22 °C for 0–30 min (Schröder et al., 1987b). After centrifugation (20000*g*, 10 min), the RNA released into the supernatant was detected as described below. In some experiments, the reaction mixtures were preincubated with insulin, EGF, or cyclic nucleotides in the absence of ATP (10 min, 22 °C) before addition of ATP.

RNA efflux experiments were always performed immediately after preparation of the nuclei. The nuclei (2.4×10^6/assay) were incubated for 0–60 min at 30 °C in transport medium [25 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 mM $MnCl_2$, 5 mM spermidine, 5 mM 2-mercaptoethanol, 250 mM D-glucose, and 300 μg/mL yeast RNA or 10^3 units/mL RNasin]. For measurement of ATP-dependent efflux, this suspension was supplemented with 2 mM ATP, 35 units/mL pyruvate kinase, 5 mM phospho*enol*-pyruvate, and 5 mM Na_2HPO_4. ATP-independent efflux was determined by adding 2 mM EDTA instead of ATP and the ATP regenerating system. If indicated, 6 mg/mL dialyzed cytosol protein was added to the medium used for measuring RNA efflux. Rapidly labeled total RNA and poly(A) RNA released into the postnuclear supernatant were determined by counting of radioactivity after trichloroacetic acid precipitation and selection on a poly(U) filter (mAP paper), respectively. Efflux of specific mRNA was detected by hybridization of dot blots or Northern blots of the isolated RNA to specific probes.

Preparation and RNA Efflux Measurements in Resealed NE Vesicles. Closed NE vesicles were prepared from rat liver nuclei as described (Riedel & Fasold, 1987a; Riedel et al., 1987). RNA was entrapped in the vesicles during the heparin lysis stage prior to resealing (Riedel & Fasold, 1987b). For RNA efflux measurements, the vesicles were resuspended in 150 μL of incubation buffer [50 mM Tris-HCl (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM ribonucleoside–vanadyl complexes, and 1 mM $PhMeSO_2F$] per assay, containing 2 mM ATP or 2 mM β,γ-methylene-ATP (for controls and measurements of ATP-independent efflux), and layered over a cushion of 40 μL of silicone oil ($d = 1.03$ g/mL) and 10 μL of 70% $HClO_4$, placed in an Eppendorf microfuge tube. After incubation for 0–30 min at 22 °C, reactions were stopped by centrifugation. Aliquots (140 μL) were taken from the supernatants (upper layer) and counted for radioactivity (Riedel & Fasold, 1987a,b).

RNA Blot Hybridization. Total RNA was isolated from nuclei, nuclear matrices, and incubation supernatants by the method of Cathala et al. (1983). Selection of poly(A+) RNA and poly(A−) RNA was performed by binding to the poly(U) filter (messenger affinity paper, mAP; Medac, Hamburg, FRG) as described previously (Messer et al., 1986). The actin DNA probe was prepared from the plasmid p41, which contains the mouse β-actin coding sequence cloned into the *Pst*I site of pBR322 (Alonso et al., 1986). As histone H4 DNA probe, the plasmid pMmH4Alu containing an *Alu*I fragment of the cloned mouse H4 gene (Seiler-Tuyns & Birnstiel, 1981) was used. The DNA probes were labeled by nick-translation with [α-^{32}P]dTTP to a specific radioactivity of $(6–8) \times 10^7$ cpm/μg of DNA as described by Rigby et al. (1977). Electrophoresis of the denatured RNA samples on agarose gels, blot-transfer to nitrocellulose, and hybridization to the ^{32}P-labeled DNA probes were done as described previously (Messer et al., 1986). The dot blot assay was a modification (Schröder et al., 1988a) of the method of White and Bancroft (1982). Exposition of the dried nitrocellulose filters to Kodak XAR-5 X-ray film backed by a screen at −70 °C was for 12–72 h.

Enzyme Assays. Nuclear envelope NTPase activity was determined as described previously (Schröder et al., 1986b) using [γ-^{32}P]ATP or [γ-^{32}P]GTP as substrate. In some assays, 20 μM poly(A) was added; at this concentration, the NTPase is stimulated about half-maximally (Agutter et al., 1977).

Nuclear envelope associated NI- and NII-like PK activity was determined as described previously (Bachmann et al., 1984; Schröder et al., 1988a). The standard assay mixture contained, in a final volume of 100 μL, 40 μg of envelope protein in 25 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 150 mM NaCl, 10 mM NaF, 10 mM *O*-phospho-DL-serine, 1 mM 2-mercaptoethanol, and 40 μM [γ-^{32}P]ATP (2×10^3 dpm/pmol). In some assays, casein was added as exogenous substrate to 2.5 mg/mL. After incubation for 0–10 min at 22 °C, proteins were pelleted by trichloroacetic acid (5%, supplemented with 1.5% sodium pyrophosphate) precipitation, and incorporated radioactivity was determined.

Protein kinase C activity was determined in reaction mixtures (150 μL) consisting of 25 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 50 μg/mL phosphatidylserine, 0.5 μg/mL diolein, and 40 μM [γ-^{32}P]ATP (2×10^3 dpm/pmol) with or without 1 mg/mL histone type III-S as exogenous substrate. Incubation was for 5 min at 30 °C. In control assays, 1 mM EGTA was added, and $CaCl_2$ and phospholipid were omitted. Incorporation of ^{32}P into protein was determined as described before.

To measure NE PPH activity (Schröder et al., 1986c), endogenously ^{32}P-labeled envelopes (prepared as described under NI/NII-like PK assay) were resuspended in 50 mM histidine/imidazole (pH 7.3) and 1 mM $MgCl_2$. Incubations were performed for 0–30 min at 22 °C in the absence or presence of 65 μM poly(A). Reactions were terminated by trichloroacetic acid precipitation (see above), and the supernatant obtained after centrifugation (20000*g*, 15 min) was counted for radioactivity. In some assays, denatured ^{32}P-la-

beled casein was used as an exogenous substrate which was prepared as described previously (Schröder et al., 1986c).

Thiophosphorylation of NE was performed under conditions optimal for PK-NII; ATP in the reaction mixture was replaced by [γ-^{35}S]ATP (for determination of PPH activity) or nonradioactive γ-thio-ATP (for determination of NTPase activity); incubation period was 5 min. After being washed in buffer without ATP analogue, PPH and NTPase activities were determined in the absence or presence of insulin and EGF as described above.

Binding to Pore Complex Laminae. The binding of [^{3}H]-poly(A) to isolated pore complex laminae was studied essentially as described previously (Schröder et al., 1986c). The pore complex laminae were prepared from unphosphorylated or phosphorylated NEs by treatment with 2% Triton X-100 for 10 min at 0 °C (Aaronson & Blobel, 1975). Phosphorylation of the envelopes was performed either by endogenous NI- and NII-like PK or by exogenous PK-C as outlined earlier (Schröder et al., 1986c), except that some samples were phosphorylated in the presence of insulin or EGF. To prevent dephosphorylation, 10 mM NaF and 10 mM *O*-phospho-DL-serine were added to the buffer used for demembranation. After incubation with [^{3}H]poly(A) in binding buffer [25 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, and 0.5 mM 2-mercaptoethanol] for 10 min at 22 °C, the pore complex laminae were pelleted by centrifuging through a 30% sucrose cushion (Bernd et al., 1982b). Bound and unbound [^{3}H]poly(A) were determined by counting the radioactivity in the pellet and in the upper phase. Evaluation of the binding data was performed by computer analysis using the Ligand program (Munson & Rodbard, 1980).

Phosphoamino Acid Analysis. The phosphorylated p106 was extracted from polyacrylamide gels (Stralfors & Belfrage, 1983) and hydrolyzed in 6 N HCl at 110 °C under nitrogen for 2 h. The phosphorylated amino acids were analyzed by two-dimensional thin-layer electrophoresis (Hunter & Sefton, 1980). The positions of nonradioactive phosphoamino acid standards were determined by staining with ninhydrin.

Photoaffinity Labeling. Rat liver nuclear matrix or NE was resuspended in 50 mM Tris-HCl (pH 7.6), 25 mM KCl, and 5 mM $MgCl_2$ at a final concentration of 100 μg of protein/mL. After addition of 50–200 μM 8-azido[α-^{32}P]ATP (6.3 Ci/mmol) plus/minus 500 μM competitor nucleotide (ATP or GTP), the suspensions were incubated for 15 min at room temperature, with or without exposure to medium-wavelength ultraviolet light (about 500 μW/cm^2 at 300 nm; distance 4 cm). The reactions were terminated by addition of ice-cold trichloroacetic acid. After being washed twice in Tris buffer, the matrices or envelopes were resuspended in 40 μL of Na-DodSO$_4$ sample buffer, heated for 5 min at 95 °C, and electrophoresed on 10% polyacrylamide gels under denaturing conditions. The dried gels were exposed to X-ray film for 1–8 days.

Incorporation of [^{32}P]phosphate into NE proteins was determined after incubation of the envelopes with [γ-^{32}P]ATP for 3 min at 30 °C, using the reaction mixture described for NI- and NII-like PK assay. The reactions were stopped by addition of ice-cold 15% trichloroacetic acid. Protein pellets were dissolved in NaDodSO$_4$ sample buffer, heat-treated (95 °C, 5 min), and electrophoresed as above. The dried gels were subjected to autoradiography for 12–48 h at −70 °C. Autoradiograms were scanned by using an integrating densitometer (Shimadzu CS-910/C-R1A).

Electrophoresis of proteins in 10% polyacrylamide slab gels containing 0.1% NaDodSO$_4$ was done as described by Laemmli (1970). Protein was measured as described by Lowry et al. (1951) using bovine serum albumin as a standard.

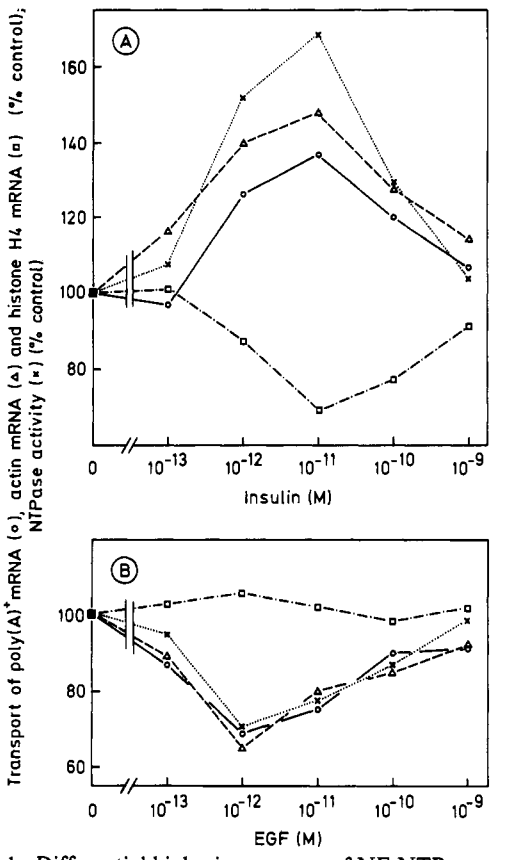

FIGURE 1: Differential biphasic responses of NE NTPase mediated nuclear efflux of poly(A+) mRNA (actin and total adenylated RNA) and poly(A−) mRNA (histone H4) to increasing concentrations of insulin and EGF. Nuclei (2.4 × 10^6/assay) from rat liver, unlabeled or prelabeled with [^{3}H]uridine, were preincubated with different concentrations of insulin (A) or EGF (B) in ATP-free transport medium (10 min, 4 °C). After addition of ATP, RNA efflux was allowed to occur for 10 min (30 °C). The released RNA was then extracted from the postnuclear supernatant and selected by binding to a poly(U) filter [mAP paper; for determination of tritium-labeled poly(A+) mRNA] (○) or analyzed by RNA dot blot hybridization to specific probes for actin (△) and histone H4 RNA (□). Autoradiograms from dot blots were quantitated by densitometric scanning using a calibration curve. Values for ATP-dependent efflux of total poly(A+) mRNA were corrected by subtracting the counts measured, under the same conditions, for ATP-independent efflux (presence of EDTA in the transport medium instead of ATP). In the absence of insulin and EGF, 4642 ± 250 dpm were found to be released in the postnuclear supernatant (10-min incubation period); this corresponds to 3.71 ± 0.20% of the total amount of nuclear radioactivity (=5.2 × 10^4 dpm/10^6 nuclei). In parallel assays, NTPase activity was determined in NE from rat liver that had been preincubated without or with insulin and EGF (×); basal activity, 0.120 ± 0.007 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$. All values are given as a percentage of control and are the means from two experiments, each done in triplicate. The SD were less than 11% [efflux of poly(A+) RNA], 15% (actin mRNA), 20% (H4 mRNA), and 6% (NTPase activity), respectively.

Statistical Evaluation. Student's *t* test was employed to determine the significance of the differences in RNA release (Sachs, 1984).

Results

Effects on Nuclear Efflux of Polyadenylated and Non-polyadenylated mRNA. As shown in Figure 1, treatment of highly purified nuclei from rat liver with insulin (panel A) or EGF (panel B) resulted in a concentration-dependent alteration in the poly(A+) mRNA efflux rate. At a concentration of 10 pM insulin, an enhancement of the amount of mRNA,

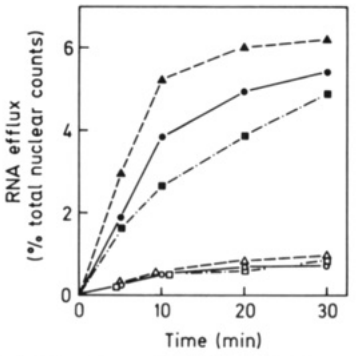

FIGURE 2: Effect of insulin and EGF on the time course of ATP-dependent efflux of poly(A+) mRNA from prelabeled rat liver nuclei. Nuclei (2.4×10^6/assay) were isolated and preincubated without (●, ○) or with 10 pM insulin (▲, △) or 1 pM EGF (■, □) in ATP-free transport medium (10 min, 4 °C). Efflux of poly(A+) mRNA was induced by addition of ATP (●, ▲, ■) and raising the temperature to 30 °C. Control assays to determine ATP-independent RNA efflux contained EDTA instead of ATP (○, △, □). The poly(A+) mRNA released into the postnuclear supernatant was detected by binding to a poly(U) filter (mAP paper) and measuring filter-bound radioactivity; it is expressed as a percentage of the total nuclear counts ($=5.1 \times 10^4$ dpm/10^6 nuclei). Results are means of three triplicate determinations; the SD were less than 9%.

transported into the postnuclear supernatant, by 37 ± 6% (mean ± SD) was detected. On the other hand, EGF at a concentration as low as 1 pM caused a decrease in mRNA efflux rate by 31 ± 5%. At higher concentrations of insulin or EGF, the mRNA efflux rates reached nearly the same levels as in untreated nuclei. The time course of nuclear poly(A+) mRNA efflux in the presence of insulin and EGF at those concentrations which were most effective is shown in Figure 2. In parallel assays, using isolated NEs, the NTPase activity, which mediates poly(A+) mRNA transport, was determined. It was found that this enzyme also responded to the two modulators with a change in specific activity paralleling the respective alterations in RNA transport rate (Figure 1A,B). At optimal concentrations, a stimulation of the NTPase activity by insulin (10 pM) to 169 ± 7% and an inhibition by EGF (1 pM) to 71 ± 3% were found; the specific activity of the NTPase in the absence of these effectors was 0.119 ± 0.005 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$. At higher concentrations of insulin and EGF, the same activity levels as in untreated cells were reached.

To determine the effect of insulin and EGF on transport of specific mRNA, we selected one poly(A)-containing mRNA (actin) and one poly(A)-free mRNA (histone H4). Northern blot analysis of both RNAs exported out of the nuclei in the absence or presence of both additives showed that they were obviously undegraded; with the actin probe, only one main band of about 2.1 kb and with the histone H4 probe a band of about 0.4 kb were detected (Figure 3A,B). Efflux of actin mRNA and histone H4 mRNA strongly depended on temperature; after incubation at 4 °C, only a very small amount of H4 mRNA and no actin mRNA were detected in the postnuclear supernatant (Figure 3C). Time kinetics of efflux of actin mRNA and H4 mRNA from untreated nuclei both in the absence and in the presence of ATP are shown in Figure 4. Efflux of histone H4 mRNA, but not of actin mRNA, was observed both in ATP-containing and in ATP-free (EDTA-containing) transport medium, consistent with previous results (Schröder et al., 1989a). Densitometric evaluation of the autoradiograms of dot blot experiments, performed after incubation of nuclei without or with 10 pM insulin or 1 pM EGF, with an integrating densitometer revealed that the initial rate of transport (0–10 min) of actin mRNA in the presence

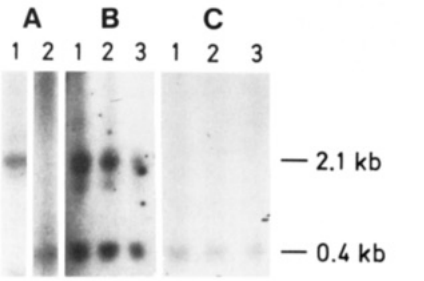

FIGURE 3: Northern blot analysis of actin mRNA and histone H4 mRNA released from isolated rat liver nuclei in the absence and presence of insulin and EGF. Nuclei were preincubated for 10 min at 4 °C in ATP-free transport medium without (A1, A2, B1, and C1) or with 10 pM insulin (B2 and C2) or 1 pM EGF (B3 and C3). Then ATP and an ATP-regenerating system were added, and incubation was continued for 20 min at 30 °C (A and B) or 4 °C (C). The RNA, released from 7.2×10^6 nuclei, was isolated, size-separated by electrophoresis on agarose, and blot-transferred to nitrocellulose. Blots were hybridized either to the ^{32}P-labeled actin (A1) or histone H4 DNA probe (A2) alone or to a mixture of both probes (B and C).

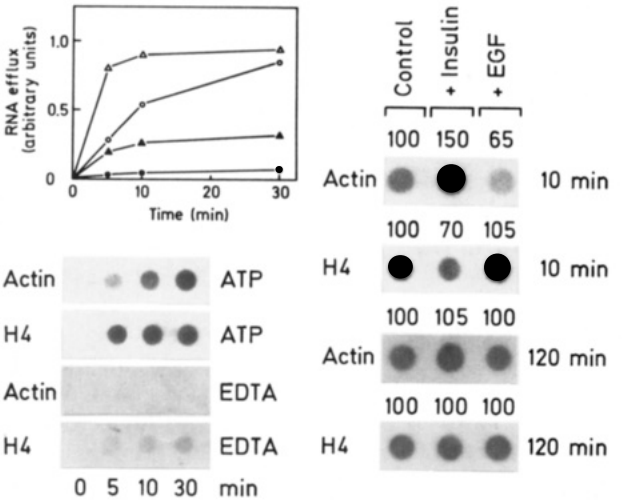

FIGURE 4: Differential effect of EGF and insulin on efflux of actin mRNA and histone H4 mRNA from isolated rat liver nuclei. The left side shows time kinetics of efflux of actin mRNA and H4 mRNA in the presence of ATP (=ATP-dependent efflux) or EDTA (=ATP-independent efflux) in the external transport medium. Dot blots of total RNA (bottom), isolated from the postnuclear supernatant (2.4×10^6 nuclei/assay) after different incubation periods, were hybridized to the ^{32}P-labeled actin and histone H4 specific probes. The autoradiograms were evaluated densitometrically. (Top) Resulting time courses of ATP-dependent (○, △) and ATP-independent (●, ▲) efflux of actin mRNA (○, ●) and H4 mRNA (△, ▲)(given in arbitrary units; efflux of RNA in the presence of ATP per 1 h was set at 1). (Right) Dot blot analysis of RNA, released from nuclei after incubation for 10 or 120 min in ATP-containing transport medium, supplemented with 10 pM insulin or 1 pM EGF. The numbers give the relative amounts of RNA released (actin or H4) in percent of control (absence of insulin and EGF; =100%).

of insulin increased by about 50% and that in the presence of EGF decreased by about 35% (Figure 4). In the case of poly(A)-free histone H4 mRNA, however, a decrease of the initial efflux rate by about 30% in the presence of insulin and a small increase by about 5% in the presence of EGF were detected. However, after a prolonged incubation period (120 min) in ATP-containing transport medium, nearly equal amounts of actin and H4 mRNA were found in the postnuclear supernatant, indicating that insulin and EGF modulate only the rate and not the maximal extent of RNA efflux (Figure 4). Figure 1A,B summarizes the data on the effects of different concentrations of insulin and EGF on the amount of actin mRNA and histone H4 mRNA appearing in the postnuclear supernatant after an incubation period of 10 min.

Table I: Effects of Insulin and EGF on Poly(A+) mRNA Release from Demembranated Nuclei and Nuclear Matrices and on PK-Dependent Phosphorylation of Detergent-Treated NE (=Pore Complex Laminae)[a]

addition	poly(A+) mRNA release[a]			PK-NII act., Triton-treated NE[c] [nmol of phosphate incorporated min^{-1} (mg of protein)$^{-1}$]	PK-C act., Triton-treated NE + PK-C[c] [pmol of phosphate incorporated min^{-1} (mg of protein)$^{-1}$]
	untreated nuclei (% total nuclear counts)	Triton-treated nuclei[b] (% total nuclear counts)	nuclear matrices (% total matrix counts)		
none	3.79 ± 0.26[d]	4.12 ± 0.25	18.2 ± 1.3	0.52 ± 0.02	3.3 ± 0.2
insulin, 10 pM	5.11 ± 0.21[e]	4.05 ± 0.32[f]	17.9 ± 1.1[f]	0.54 ± 0.03[f]	3.2 ± 0.1[f]
EGF, 1 pM	2.94 ± 0.21[e]	4.11 ± 0.21[f]	18.6 ± 1.5[f]	0.51 ± 0.02[f]	3.3 ± 0.2[f]

[a] Measurements of ATP-dependent release of poly(A+) mRNA from untreated nuclei, demembranated nuclei, and nuclear matrices were performed as described under Materials and Methods (incubation period, 10 min); values are given as the percentage of total nuclear counts (=5.4 × 10^4 dpm/10^6 nuclei) or total matrix counts (=3.0 × 10^4 dpm/10^6 matrices). [b] Nuclei were freed from nuclear membranes by treatment with 1% Triton X-100 in ATP-free transport medium (10 min, 4 °C), followed by one washing cycle in this solution without detergent. [c] Treatment of NE with Triton X-100 was performed as described by Aaronson and Blobel (1975). Autophosphorylation of the resulting pore complex laminae by endogenous NII-like PK [without poly(A)] or phosphorylation by exogenous PK-C [1.8 units/assay; incubated with the pore complex laminae in the presence of 100 μM Ca^{2+}, 20 μg/mL phosphatidylserine, and 0.02 μg/mL phorbol 12-myristate 13-acetate; see Schröder et al. (1988b)] was for 5 min at 30 °C. [d] Results are means ± SD from two experiments, each done in triplicate. [e] Significant at $p < 0.001$. [f] Not significant at $p < 0.05$.

The experiments revealed that at higher (unphysiological) concentrations of insulin or EGF, the effect of both proteins on transport disappeared; this resulted in biphasic dose–response curves, resembling those found for the efflux of total poly(A+) mRNA.

Pretreatment of the nuclei with Triton X-100, which removes the outer and part of the inner nuclear membrane, resulted in a loss of responsiveness of transport of rapidly labeled poly(A+) mRNA to insulin and EGF (Table I). Transport of mRNA from demembranated nuclei, which lack the NE NTPase (Schröder et al., 1986b) has previously been shown also to depend on ATP, but not to require hydrolysis of this nucleotide (Schröder et al., 1987b). Efflux of actin mRNA and H4 mRNA from membrane-depleted nuclei also did not respond to either insulin or EGF (not shown). Insulin and EGF also were found to have no influence on release of rapidly labeled poly(A+) mRNA from isolated nuclear matrix from rat liver (Table I). Similar results were obtained for ATP-caused detachment of matrix-bound actin mRNA and histone H4 mRNA [data not shown; in contrast to actin mRNA, poly(A)-free histone H4 mRNA was found to be associated with nuclear matrix only to a very small extent; cf. also Bandyopadhyay et al. (1986) and Schröder et al. (1989a)].

Effect on RNA Efflux from Closed NE Vesicles. Resealed NE vesicles containing entrapped exogenous RNA represent a useful model to investigate RNA translocation across the NE, independent from RNA binding to intranuclear binding sites and its release from these sites (Riedel & Fasold, 1987a,b; Riedel et al., 1987). Efflux of poly(A+) RNA has been shown to be ATP dependent, similar to efflux from isolated nuclei, and to involve a NE NTPase activity, which is enriched in vesicle preparations (Riedel et al., 1987). The specific activity of this enzyme was determined to be 0.052 ± 0.007 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$. In previous studies, it was demonstrated that ATP-dependent efflux of poly(A+) mRNA from the vesicles is not caused by release of adsorbed material from the vesicle surface or by RNA degradation (Riedel et al., 1987; unpublished results). In Figure 5A, it is shown that purified polysomal poly(A+) mRNA entrapped in NE vesicles was released in the extravesicular space in an ATP-dependent manner, while export of poly(A−) mRNA, obtained after removal of the poly(A) tail by endoribonuclease IV, was not stimulated by ATP (B). Pretreatment of the vesicles with insulin (10 pM) caused an 1.3-fold increase in the initial efflux rate of poly(A+) mRNA (5-min incubation period), while efflux of poly(A−) mRNA was unaffected (Figure 5). Addition of EGF, on the other hand, decreased ATP-dependent export of poly(A+) mRNA (to about 70%

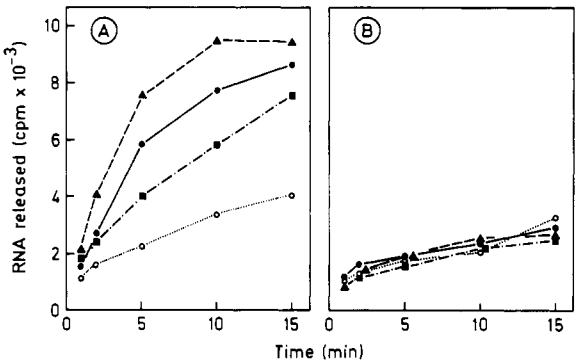

FIGURE 5: Effect of insulin and EGF on efflux of polysomal poly(A+) mRNA and poly(A−) mRNA from resealed NE vesicles. Tritium-labeled polysomal poly(A+) mRNA (11 380 cpm/assay) and poly(A−) mRNA, obtained after endoribonuclease IV digestion (10 210 cpm/assay), were trapped in the vesicles. Efflux of poly(A+) mRNA (A) and poly(A−) mRNA (B) was determined after preincubation (10 min; 4 °C) of the vesicles without (●, ○) or with 10 pM insulin (▲) or 1 pM EGF (■) in nucleotide-free incubation buffer. ATP-dependent efflux was initiated by addition of 2 mM ATP (●, ▲, ■); assays to determine ATP-independent efflux contained 2 mM β,γ-methylene-ATP instead of ATP (○). Means of five separate experiments are given; the SD were less than 13%.

at 1 pM) but not ATP-independent efflux of poly(A−) mRNA (Figure 5). At 30–60 min, efflux of poly(A+) mRNA reached a plateau (release of 90–95% of the entrapped RNA; not shown in Figure 5). In parallel assays, it was determined that, at the same concentrations of insulin and EGF, the NE NTPase activity, present in the vesicles, is increased to 145% and decreased to 81%, respectively.

Effects on Phosphorylation and Dephosphorylation of NE and on the Affinity of the Poly(A) Binding Component. In a previous paper, we demonstrated that NE preparations contain both an NI/NII-like PK and a PK-C activity (Schröder et al., 1988b). Phosphorylation of rat liver NE by endogenous NII-like PK resulted in incorporation of phosphate into several polypeptide bands; one group of major phosphorylated bands was in the range of nuclear lamins with M_r 60K–70K [Figure 6, lane b; see also Schröder et al. (1988b)]. The phosphorylation by this enzyme has been shown to be inhibited by poly(A) and poly(G) (McDonald & Agutter, 1980; Bachmann et al., 1984; Schröder et al., 1988b); among the NE phosphoproteins, one minor protein (M_r 106K) seemed to be more sensitive to inhibition of phosphorylation by poly(A) than the others. This protein, which is assumed to represent the poly(A)-recognizing mRNA carrier, was also phosphorylated by PK-C (Schröder et al., 1988b). In Figure 7, it is shown that incorporation of phosphate into this protein, excised

Table II: Effect of Insulin and EGF on NE-Associated PK and PPH Activities in the Absence or Presence of Poly(A)[a]

addition	PK act. [nmol of phosphate incorporated min^{-1} (mg of protein)$^{-1}$][b]		PPH act. [nmol of phosphate liberated min^{-1} (mg of protein)$^{-1}$][b]	
	−poly(A)	+poly(A)	−poly(A)	+poly(A)
none	0.038 ± 0.002	0.029 ± 0.002	0.43 ± 0.03	0.71 ± 0.04
insulin				
1 pM	0.037 ± 0.002	0.029 ± 0.001	0.48 ± 0.04	0.77 ± 0.05
10 pM	0.034 ± 0.003	0.026 ± 0.001	0.69 ± 0.05[c]	1.17 ± 0.09[c]
100 pM	0.035 ± 0.002	0.028 ± 0.001	0.49 ± 0.02[c]	0.80 ± 0.05[c]
EGF				
0.1 pM	0.040 ± 0.001	0.030 ± 0.001	0.42 ± 0.03	0.70 ± 0.05
1 pM	0.048 ± 0.003[c]	0.035 ± 0.002[c]	0.42 ± 0.03	0.71 ± 0.04
10 pM	0.041 ± 0.001	0.031 ± 0.002	0.43 ± 0.02	0.69 ± 0.03

[a] Endogenous NI/NII-like PK activity of unphosphorylated NE (without addition of exogenous casein substrate) and PPH activity of ^{32}P-phosphorylated envelopes (hydrolysis of endogenous phosphoproteins) were determined in the absence or presence of 65 μM poly(A) (with respect to phosphate). Incubations in the presence of different concentrations of insulin or EGF were performed for 5 min (PK) or 10 min (PPH assay). [b] Results are means ± SD from quadruplicate experiments. [c] Significant stimulation compared with control ($p < 0.05$).

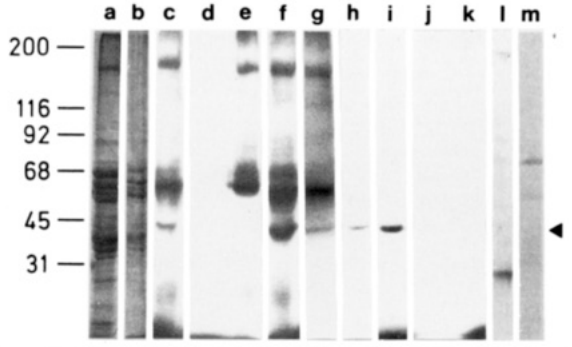

FIGURE 6: Photoaffinity labeling of rat liver NE and of purified NTPase with 8-azido-ATP. Exposition of NE suspension (20 μg of protein/assay) (lanes c–g) and of purified NTPase (4 μg/assay) (lanes i–k) with 100 μM 8-azido[α-^{32}P]ATP to medium-wavelength ultraviolet light was performed as described under Materials and Methods. Reactions were done both in the absence (lanes c and i) and in the presence of competitor ATP (lanes d and j) or GTP (lanes e and k) (500 μM each). In lane f, the reaction mixture contained 20 μM poly(A), and in lane g, 20 μM poly(C). In parallel assay, NEs (15 μg) were autophosphorylated with [γ-^{32}P]ATP under conditions optimal for NI/NII-like PK (lane b). Lane l, purified NTPase (2 μg) phosphorylated by isolated PK-NII (0.4 μg); lane m, purified NTPase (3 μg) phosphorylated by isolated PK-C (0.2 μg). The samples were subjected to electrophoresis in $NaDodSO_4$/10% polyacrylamide gels. Shown are the autoradiograms. Lane a shows a Coomassie blue stained gel of (b–g) (=NE preparation); a silver-stained gel of the purified NTPase (i–k) is shown in lane h. Numbers on the left side are $M_r \times 10^{-3}$. Arrowhead, NE NTPase.

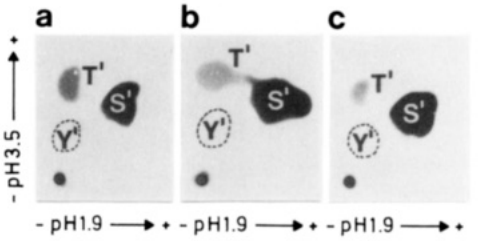

FIGURE 7: Phosphoamino acid analysis of extracted p106 phosphorylated by endogenous NE NI/NII-like PK in the absence or presence of insulin or EGF. Autophosphorylation of NE (5 min, 22 °C) with [γ-^{32}P]ATP under conditions optimal for PK-NI/NII in the absence (a) or presence of 10 pM insulin (b) or 1 pM EGF (c) was performed as described under Materials and Methods. Subsequently, the samples were subjected to $NaDodSO_4$/10% polyacrylamide gel electrophoresis, and protein phosphorylation was visualized by autoradiography. The ^{32}P-labeled p106 (106-kDa band) was then excised from the gel, eluted, and hydrolyzed under acidic conditions. The phosphorylated amino acids were analyzed by two-dimensional thin-layer electrophoresis followed by autoradiography. S′, phosphoserine; T′, phosphothreonine; Y′, phosphotyrosine (position of unlabeled standard indicated by dashed circle); (O) start.

from polyacrylamide gels, occurred predominantly into serine residues, both under phosphorylation conditions optimal for PK-NII and PK-C. Preincubation of NE with insulin or EGF did not result in a change in the relative intensities of the phosphoserine and phosphothreonine spots (Figure 7). No incorporation of phosphate into tyrosine could be detected, both in the absence and in the presence of the effectors, indicating that a tyrosine-specific PK is not involved in phosphorylation of p106. In addition, we obtained no hints that both effectors affect selectively the phosphorylation of particular NE polypeptides, because preincubation of NE with 10 pM insulin or 1 pM EGF resulted in similar changes in phosphorylation of obviously all NE phosphoprotein bands (not shown). Autophosphorylation of membrane-depleted NEs (obtained by treatment with Triton X-100), which contain the NII-like PK activity but lack PK-C activity (Schröder et al., 1988b), under conditions optimal for PK-NII failed to be affected by insulin or EGF (Table I). This was not due to the absence of PK-C, because addition of isolated PK-C plus phospholipid did not restore the ability of both proteins to alter NE phosphorylation (Table I). The most plausible explanation seems to be the absence of insulin and EGF receptors after pretreatment of nuclei with Triton X-100.

The insulin-caused decrease in incorporation of [^{32}P]phosphate into NE protein seems not to be due to an inhibition of NE PK activities but rather to be due to an enhancement of NE PPH activity by the hormone. On the other hand, the enhanced phosphorylation of NEs in the presence of EGF seems to be caused by an increase in the phosphorylation reaction itself. These conclusions have been drawn from the results shown in Table II. It was found that the NE PPH activity was significantly ($p < 0.005$) enhanced in the presence of picomolar concentrations of insulin but remained obviously unchanged in the presence of EGF [both in the absence and in the presence of poly(A)]. As further proof that the NE PPH activity is the primary target of the action of insulin on the mRNA translocation system, NEs were thiophosphorylated with γ-thio-ATP. Thiophosphorylated proteins are known to be phosphatase resistant (Cassel & Glaser, 1982). Autoradiograms of $NaDodSO_4$ gels of NEs that had been thiophosphorylated with [γ-^{35}S]ATP revealed the same pattern of labeled polypeptide bands as NE phosphorylated with [γ-^{32}P]ATP (not shown). The thiophosphorylated envelopes were found to be less prone to dephosphorylation by NE PPH than the phosphorylated ones; the PPH activity amounted to 0.063 ± 0.004 nmol of thiophosphate min^{-1} (mg of protein)$^{-1}$ (control), 0.065 ± 0.005 nmol min^{-1} mg^{-1} (presence of 10 pM insulin), and 0.064 ± 0.004 nmol min^{-1} mg^{-1} (presence of 1 pM EGF)(means ± SD from three independent determina-

Table III: Influence of Insulin and EGF on Phosphorylation-Related Enhancement of Poly(A) Binding to Isolated Pore Complex Laminae[a]

pore complex laminae	K_a (μM^{-1})	no. of binding sites [nmol of poly(A) phosphate/mg of protein]
unphosphorylated	4.52	2.3
phosphorylated by PK-NII	14.31	2.4
plus 10 pM insulin	6.92	2.2
plus 1 pM EGF	18.02	2.4
phosphorylated by PK-C	12.12	2.4
plus 10 pM insulin	7.31	2.3
plus 1 pM EGF	17.98	2.4

[a] NEs were phosphorylated by endogenous NII-like PK or PK-C in the absence or presence of 10 pM insulin or 1 pM EGF for 10 min at 22 °C. Pore complex laminae were prepared from the envelopes and incubated with increasing concentrations of [^{3}H]poly(A) as described under Materials and Methods. Binding experiments were performed in triplicate, and binding data were computed from Scatchard plots using the Ligand program (Munson & Rodbard, 1980).

Table IV: Alteration of the Kinetic Parameters of NE NTPase and of Its Stimulation by Poly(A) in the Presence of Insulin and EGF[a]

addition	V_{max} [μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$]	K_m (mM)	% change in act.[b] after addition of poly(A)	% change in act.[b] after addition of poly(C)
none	0.19	0.41	166	101
insulin				
1 pM	0.23	0.42	159	105
10 pM	0.42	0.40	181	100
100 pM	0.28	0.40	171	96
EGF				
0.1 pM	0.18	0.43	165	102
1 pM	0.14	0.41	167	95
10 pM	0.20	0.42	165	98

[a] NE NTPase activity was measured after addition of increasing concentrations of insulin or EGF in the absence or presence of 20 μM poly(A) or poly(C) (with respect to phosphate). For determination of V_{max} and K_m, 150 mM KCl was added to the reaction mixtures in order to obtain linear Lineweaver–Burk plots (Agutter et al., 1979), and poly(A) was omitted. [b] The enzyme activity in the absence of poly(A) or poly(C) was set at 100%.

tions). Using envelopes that had been thiophosphorylated with nonradioactive γ-thio-ATP, only a little stimulatory effect of insulin on NTPase activity was determined, while the inhibition of the enzyme activity by EGF was not affected.

From these results, a decrease in the net phosphorylation of NE phosphoproteins should occur in the presence of insulin, resulting in a lower poly(A) binding affinity of the NE mRNA binding site. Addition of EGF should cause the opposite effect. These predictions could be confirmed in poly(A) binding experiments, using the pore complex lamina fraction of NE [treatment with Triton X-100 removes nearly all unspecific poly(A) binding sites; McDonald & Agutter, 1980]. Pore complex laminae have been already demonstrated to contain only one class of poly(A) binding sites (McDonald & Agutter, 1980; Bernd et al., 1982b; Schröder et al., 1988b). As summarized in Table III, the affinity constant (K_a) of this site for poly(A) was found to increase after phosphorylation of the envelopes with both endogenous NII-like PK and PK-C, while the total number of binding sites remained unchanged. Phosphorylation of the NE under PK-NII or PK-C conditions in the presence of picomolar concentrations of insulin or EGF, however, resulted in a significant, differential change of the poly(A) binding affinity of the pore complex laminae, resulting in lower (in the case of insulin) or higher K_a values (EGF) (Table III). The total number of poly(A) binding sites did not change in the presence of both additives. Addition of a 10-fold excess of unlabeled poly(A) at that concentration of [^{3}H]poly(A) (=0.22 μM) which corresponds to the K_d in assays using unphosphorylated NE reduced the amount of bound [^{3}H]poly(A) to about 0.2 nmol/mg of NE protein, while addition of unlabeled poly(C) at the same concentration ratio only resulted in a small decrease of about 20% (to 0.92 nmol/mg). This result indicates that the NE poly(A) binding sites display a high specificity for poly(A).

Effects on NTPase Activity and Its Stimulation by Poly(A). Phosphorylation and dephosphorylation of NE have been shown to modulate not only poly(A) binding affinity of the mRNA binding site of the translocation apparatus but also NE NTPase activity, providing the energy for mRNA transport (Agutter et al., 1976; Bernd et al., 1982b; Schröder et al., 1988b). This enzyme has been shown to be specificially stimulated by poly(A) if it is bound to NE (Bernd et al., 1982a); the solubilized and purified enzyme does not respond to poly(A) (Schröder et al., 1986b), most likely due to the absence of the NE poly(A) binding site which seems to be intimately coupled with the enzyme. Table IV shows the effect of insulin and of EGF on some kinetic parameters of the NE-bound NTPase. The insulin-caused increase in specific activity of NE NTPase was found to be due to an increase in the maximum catalytic rate (V_{max}); the apparent K_m of the enzyme did not change in the presence of the hormone (Table IV). Treatment with EGF resulted in a decrease of V_{max}, also without affecting K_m (Table IV). While the activity of the structure-bound enzyme was significantly affected by insulin and EGF, the homogeneous NTPase did not respond at all (not shown). Interestingly, the extent of poly(A)-caused stimulation of the NE-bound enzyme (the homogeneous enzyme is not affected by the polymer) was higher in envelopes which had been preincubated in the presence of insulin at that concentration which was also maximally stimulatory for NTPase activity in the absence of poly(A). On the other hand, the capacity of the NTPase to be stimulated by poly(A) was not significantly altered in the presence of EGF (Table IV). Poly(C) displayed no stimulatory effect on the enzyme activity (Table IV).

Previously we established that NE NTPase activity and mRNA efflux are inhibited by tumor promoters that activate PK-C (Schröder et al., 1988a,b). Now we determined that both the NTPase activity and mRNA efflux rate are enhanced in the presence of inhibitors of both PK-C (H-7 and staurosporine) and PK-NII (heparin). In the presence of these compounds, the insulin-caused increase of NE NTPase activity (to 172 ± 6% at 10 pM insulin) was found to be enhanced to 262 ± 11% (20 μg/mL heparin), 218 ± 10% (10 μM H-7), and 227 ± 11% (0.05 μg/mL staurosporine), respectively. In the absence of insulin, these inhibitors stimulated the NTPase activity to 138 ± 4%, 120 ± 5%, and 127 ± 4%, respectively. The decrease (to 69 ± 2%) of NE NTPase activity by EGF (1 pM), on the other hand, was found to be abolished. (Results are means ± SD from two experiments, each done in triplicate.) Similar changes were observed for NTPase-mediated poly(A+) RNA efflux (data not shown). These findings corroborate the assumption that insulin primarily acts via stimulation of NE PPH and EGF via stimulation of the NE PK activity.

Identification of the NTPase in Isolated NE. To identify the NTPase in whole, isolated NEs, photoaffinity labeling experiments with 8-azido[α-^{32}P]ATP were performed. In control experiments, it was assessed that this nucleotide analogue is hydrolyzed by the NTPase at comparable rates to ATP (not shown). As shown in the autoradiogram (lane c) in Figure

6, NEs from rat liver contain only three major photoaffinity-labeled polypeptide bands (M_r ~150K, 60K, and 42K). In the presence of excess unlabeled ATP, labeling of all bands was suppressed (lane d). However, GTP which is besides ATP, a substrate of the NE-associated and purified NTPase (Schröder et al., 1986b), competed with the labeling of only one polypeptide band (M_r 42K) (lane e); CTP and UTP, which are cleaved by the NTPase with a lower rate, were less effective (not shown). Interestingly, labeling of the 42-kDa band was increased in the presence of poly(A) (lane f), which stimulates the NTPase, but not in the presence of poly(C) (lane g) which displays no stimulatory effect (see Table IV). More striking, the band of the photoaffinity-labeled, purified NTPase (lane i) was found to coincide with that of the enzyme in whole envelopes, indicating that the 42-kDa polypeptide labeled in intact NE indeed represents the NTPase. Photolabeling of the 42-kDa band depended on UV irradiation (not shown). In parallel experiments, NEs were phosphorylated with [γ-^{32}P]ATP. The results revealed no incorporation of [^{32}P]-phosphate into the 42-kDa band (Figure 6, lane b). In addition, it was shown that the purified, homogeneous enzyme is phosphorylated by neither isolated PK-NII nor PK-C (Figure 6, lanes l and m). The phosphorylated 28-kDa band in lane l, also shown with PK-NII alone, is assumed to be a proteolytic fragment of PK-NII [M_r 72K; see Delpech et al. (1986)]. The 77-kDa phosphoprotein band visible in lane m represents the autophosphorylated PK-C.

Effect of cAMP and cGMP on mRNA Transport. The endogenous PK activities of NE from rat liver have been shown to be independent of cyclic nucleotides (Schröder et al., 1987a). Previously we determined, however, that the efficiency of the transport stimulatory protein p31 to enhance mRNA efflux from isolated nuclei is increased if it is added to the transport medium in the presence of cAMP (Schröder et al., 1986c). In addition, it has been demonstrated by Schumm and Webb (1978) that cAMP and cGMP at physiological concentrations (~10^{-6} and ~10^{-7} M, respectively) significantly enhance mRNA transport in vitro; higher concentrations were less effective (Schumm & Webb, 1978). This effect was observed only in the presence of cytosolic proteins (100000*g* supernatant) (Schumm & Webb, 1978). These results could be confirmed by us. At optimal concentrations (10^{-6} M cAMP and 10^{-7} M cGMP), the amount of poly(A+) RNA transported into the postnuclear supernatant (10-min incubation period) increased from 5.81 ± 0.46% total nuclear counts to 9.01 ± 0.83% and to 8.32 ± 0.86%, respectively (means ± SD from two experiments, each done in triplicate). (The measured transport rates are higher than in the assays without cytosolic proteins due to the presence of transport stimulatory proteins in this preparation.) In addition, it was determined that, under the same conditions (presence of cytosolic proteins), the efflux of actin mRNA was 2.2-fold (addition of cAMP) and 1.8-fold (addition of cGMP), respectively, higher than in the absence of cyclic nucleotides, while the histone H4 mRNA efflux decreased by about 20–30%. These changes in RNA efflux rates paralleled an increase in NE NTPase activity from 0.12 ± 0.01 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$ (unstimulated control) to 0.20 ± 0.01 and 0.19 ± 0.01 μmol min^{-1} mg^{-1}, respectively. However, the increase in poly(A+) mRNA transport rate and NE NTPase activity in the presence of insulin seems not be caused by alterations in the levels of cAMP and cGMP for the following reasons. First, both nucleotides displayed no effect on phosphorylation of isolated envelopes; second, addition of the phosphodiesterase inhibitor theophylline (10 mM) did not enhance the stimulation caused by insulin; third, addition of NaF (a stimulator of adenylate cyclase) or NaN_3 (a stimulator of guanylate cyclase) did not stimulate but inhibited the NE NTPase activity and poly(A+) mRNA transport (data not shown).

Discussion

The mRNA translocation system within the NE pore complex is thought to be composed of both structural (e.g., lamin B; Baglia & Maul, 1983), signal receptor-like [e.g., poly(A)-recognizing mRNA carrier; Schröder et al., 1987a, 1988b], and enzymatic components (NTPase, NII-like PK, PK-C, and PPH; Agutter et al., 1976; Bachmann et al., 1984; Schröder et al., 1986b, 1988b). These interacting elements are prone to modulation by a series of physiological effectors, such as mRNA transport stimulatory proteins (Schröder et al., 1986a,c), lectins (Baglia & Maul, 1983; Kljajic et al., 1987), amino acids (Schröder et al., 1989b), hormones (Bernd et al., 1982b, 1983; Schumm & Webb, 1981; Purrello et al., 1982), and growth factors (this study). In a series of studies, it has been established that insulin is able to interact with the NE mRNA transport apparatus by direct binding to the NE receptors (Goldfine & Smith, 1976; Vignery et al., 1978; Goidl, 1979; Schumm & Webb, 1981; Purrello et al., 1982, 1983). Evidence from fluorescence photobleaching experiments indicates that EGF, which binds to isolated nuclei (Johnson et al., 1980), is also able to modulate macromolecular exchange processes across the NE (Schindler & Jiang, 1987). In the present work, the effect of these modulators on nucleocytoplasmic transport of both one poly(A)-containing mRNA (actin) and one poly(A)-free mRNA (histone H4) was studied.

Completing previous findings (Schumm & Webb, 1981; Purrello et al., 1983), the presented results suggest that the stimulatory effect of insulin is restricted to poly(A)-containing mRNAs, such as actin mRNA, only, and is not observed in the case of poly(A)-free histone H4 mRNA. More precisely, insulin even caused an opposite effect on histone H4 mRNA efflux as that obtained with actin mRNA. EGF, on the other hand, inhibited the transport of poly(A)-containing actin mRNA and caused only a slight increase in H4 mRNA transport. Although both effectors (insulin and EGF) alter mRNA efflux through affecting NE NTPase activity, their mode of action appears to be different. Insulin was found to stimulate the NE PPH activity, which in turn dephosphorylates the NE poly(A) binding site, p106 (=mRNA carrier). Hence, the NE NTPase activity is increased (because its activity is inversely correlated with the phosphorylation of p106; Schröder et al., 1987a). The enhancement of NTPase activity seems to be caused by an increase in number of active enzyme molecules and not to be caused by an increase in affinity of the enzyme to its substratum (ATP or GTP). This conclusion was drawn from the finding that only V_{max} was enhanced in the presence of insulin, while the apparent K_m (for ATP) remained unchanged. On the contrary, EGF does not affect PPH but stimulates PK and thus inhibits, via phosphorylation of the carrier, the NTPase. The effect of insulin (and of EGF) is not due to a postranslational modification of the NTPase protein itself, as proposed previously (Purrello et al., 1983), but seems to be due to an insulin-caused decrease (EGF-caused increase) in the extent of phosphorylation of the poly(A)-binding mRNA carrier. This change in the phosphorylation stage of the carrier results in turn in a stimulation (inhibition) of the NTPase through coupling within the intact NE structure. This conclusion is based on the following findings. (i) The NTPase is not a phosphoprotein; it is phosphorylated neither by endogenous PK activities in whole envelopes (Clawson et al., 1984; see also experiments in this paper) nor

by isolated kinases (PK-NII or PK-C) in the purified stage (this paper). (ii) Only the structure-bound and not the purified NTPase is modulated by the 3′-poly(A) sequence of mRNA (Bernd et al., 1982a; Schröder et al., 1986b), because this sequence binds to the mRNA carrier and not to the NTPase (Schröder et al., 1987a, 1988b). (iii) The purified NTPase does not show any response to insulin (or EGF), while the poly(A) binding affinity of the mRNA carrier embedded in the envelope is significantly changed by these proteins (this paper).

The increase in PPH-mediated dephosphorylation of the NE protein by insulin was found to be accompanied by a decrease in affinity of the resulting envelopes to bind poly(A). The rise in NE phosphorylation caused by EGF, on the other hand, paralleled with an enhancement in NE poly(A) binding affinity. These results agree with our previous findings (Bernd et al., 1982b) that dephosphorylated envelopes display a lower affinity to poly(A) than the phosphorylated ones. Interestingly, the value of the dissociation constant of poly(A) binding to dephosphorylated NE (K_d = 0.22 μM; with respect to phosphate content) is similar to the estimated intracellular concentration of high-abundance mRNAs ($\approx$1 μM; Sippel et al., 1977). Therefore, the observed insulin-caused increase in transport rate of higher abundant poly(A+) mRNAs, such as actin mRNA, might partially occur at the expense of lower abundance messengers, which are not able to bind efficiently to the dephosphorylated carrier. The opposite may be the case for EGF. Future studies must show whether nucleocytoplasmic transport of low-abundance mRNAs is enhanced after addition of insulin (or decreased after addition of EGF) to the same extent as the efflux of abundant actin mRNA.

In contrast to the effect of insulin and EGF, that results in a modulation of the poly(A+) mRNA translocation system, the mechanism underlying the insulin- and EGF-caused change in histone poly(A−) mRNA transport rate is entirely unknown. The effect on histone mRNA transport might be explained by simple competition between poly(A+) mRNA and poly(A−) mRNA for a common NE binding site, which is not identical with the mRNA carrier (p106), recognizing only adenylated messengers. The existence of such sites has been suggested previously during our studies on the effect of a monoclonal antibody, which recognizes a p60 nuclear pore complex antigen, on transport of both mRNA species (Schröder et al., 1989a). Another possibility might be that the insulin-caused enhancement of NE PPH activity or the EGF-caused stimulation of NE PK activity affects the function of some other NE component, which is involved in transport of histone poly(A−) mRNA. This assumption is supported by our recent data (unpublished results) which revealed that the NE contains an RNA unwindase activity, which is apparently involved in transport of histone mRNA via recognizing its 3′-terminal hairpin loop structure (Hentschel & Birnstiel, 1981).

Acknowledgments

We thank Dr. M. Buckingham (Institut Pasteur, Département de Biologie Moléculaire, Paris, France) and Dr. D. Schümperli (Universität Zürich, Switzerland) for the gift of the DNA clones.

Registry No. EGF, 62229-50-9; NTPase, 9075-51-8; PPH, 9025-75-6; insulin, 9004-10-8.

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