& Snyder, S. H., Eds.) Plenum Press, New York.
Fewtrell, C. M. S. (1976) *Neuroscience 1*, 249–273.
Gill, E. W., & Rang, H. P. (1966) *Mol. Pharmacol. 2*, 284–297.
Heilbronn, E., & Bartfai, T. (1978) *Prog. Neurobiol. (Oxford) 11*, 171–188.
Herron, G. S., & Schimerlik, M. I. (1983) *J. Neurochem. 41*, 1414–1420.
Herron, G. S., Miller, S., Manley, W.-L., & Schimerlik, M. I. (1982) *Biochemistry 21*, 515–520.
Krebs, K. G., Heusser, D., & Wimmer, H. (1969) in *Thin Layer Chromatography* (Stahl, E., Ed.) p 883, Springer-Verlag, Berlin.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Moreno-Yanes, J. A., & Mahler, H. R. (1980) *Biochem. Biophys. Res. Commun. 92*, 610–617.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Peterson, G. L., & Schimerlik, M. I. (1984) *Prep. Biochem. 14*, 33–74.
Schimerlik, M. I., & Searles, R. P. (1980) *Biochemistry 19*, 3407–3413.
Schmidt, J., & Raftery, M. A. (1973) *Anal. Biochem. 52*, 349–354.
Venter, J. C. (1983) *J. Biol. Chem. 258*, 4842–4848.
Yamamura, H. I., & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1725–1729.

Photoaffinity Labeling of the Major Nucleosidetriphosphatase of Rat Liver Nuclear Envelope†

Gary A. Clawson,* C. H. Woo, Jane Button, and Edward A. Smuckler

ABSTRACT: We employed the photoaffinity probe 8-azidoadenosine 5′-triphosphate (aATP) to identify the nuclear envelope (NE) nucleosidetriphosphatase activity (NTPase) implicated in control of RNA transport. The photoprobe was hydrolyzed at rates comparable to those for ATP, with a Michaelis constant of 0.225 mM. Photolabeling was dependent upon UV irradiation (300-nm max) and was not affected by quercetin. Unlabeled ATP or GTP competed with [^{32}P]aATP in photolabeling experiments, and UTP was a less effective competitor, paralleling the substrate specificity of the NTPase. Incubation of NE with aATP led to a UV, time, and concentration dependent irreversible inactivation of NTPase. The inactivation could be blocked by ATP or GTP. Polyacrylamide gel electrophoresis and autoradiography of photolabeled NE showed selective, UV-dependent labeling of a 46-kDa protein with both [γ-^{32}P]aATP and [α-^{32}P]aATP. This band was not labeled with [γ-^{32}P]ATP. Since the NE NTPase implicated in RNA transport is modulated by RNA, we examined the effects of RNA on the labeling process. Removal of RNA from the NE preparations (by RNase/DNase digestion) reduced NTPase by 30–40% and eliminated photolabeling of the 46-kDa band. Addition of yeast RNA to such preparations increased NTPase activity to control levels and selectively reinstated photolabeling of the 46-kDa band. These results suggest that the 46-kDa protein represents the major NTPase implicated in RNA transport.

Transfer of prelabeled RNA from nuclei to a surrogate cytoplasm is an energy-dependent process (Ishikawa et al., 1969; Raskas, 1971; Racevskis & Webb, 1974; Clawson et al., 1978; Jacobs & Birnie, 1982). Current evidence suggests the energy is provided by phosphate bond cleavage of di- or triphosphate nucleotides by a nuclear envelope (NE) triphosphatase (Agutter et al., 1979b; Clawson et al., 1980a,b; Purrello et al., 1982; Murty et al., 1983; Baglia & Maul, 1983). Furthermore, the activity of this enzyme is modulated by RNA (Agutter et al., 1979b; Clawson et al., 1980a). The nuclear envelope is a unique double-membrane structure containing specialized pore lamin components (Dwyer & Blobel, 1974; Comings & Okada, 1976; Maul, 1977; Gerace et al., 1978; Krohne et al., 1978; Berezney, 1979; Shaper et al., 1979; Unwin & Milligan, 1982), and its preparations consists of a distinct and limited array of polypeptides (Franke, 1974; Lam & Kasper, 1979; Richardson & Maddy, 1980b; Shelton et al., 1980) when separated by polyacrylamide electrophoresis under denaturing conditions. However, the identity of the protein(s) responsible for the nucleosidetriphosphatase (NTPase) activity (activities) has not been established. We used 8-azidoadenosine 5′-triphosphate (aATP) as a selective photoaffinity agent (Haley & Hoffman, 1974; Koberstein et al., 1976; Wagenvoord et al., 1977; Shia & Pilch, 1983; Hollemans et al., 1983) to mark the enzyme(s). We observed photolabeling of a 46-kDa NE protein, which was modulated by RNA, and suggest that this band contains the major NTPase implicated in RNA transport.

Materials and Methods

Nuclei were prepared from male Sprague-Dawley rats by the technique of Blobel & Potter (1966): 0.5 mM phenylmethanesulfonyl fluoride (PMSF) was included in the 2.3 M sucrose buffer cushion and in all subsequent preparative steps. NE were isolated by a modification of the technique described by Monneron (1974), with PMSF included in the gradient. NTPase activity was determined as described (Clawson et al., 1980a). To remove RNA from NE preparations, purified NE were resuspended in TKM buffer (50 mM Tris-HCl [tris(hydroxymethyl)aminomethane hydrochloride] (pH 7.6), 25 mM KCl, 5 mM $MgCl_2$) plus 0.5 mM PMSF, DNase I (Miles) was added to 10 μg/mL and RNase A was added to 10 μg/mL, and this suspension was incubated for 20 min at room temperature; the resulting suspension was layered over

† From the Department of Pathology, University of California, San Francisco, California 94143. *Received November 3, 1983.* This work was supported by Grant CA21141 from the NIH.

0006-2960/84/0423-3501$01.50/0

the same buffer also containing 0.25 M sucrose. NE were harvested by centrifugation at 40000*g* for 15 min at 4 °C in a Beckman 50 Ti rotor.

For photoaffinity labeling experiments, 50–100 μg of NE protein were resuspended in 8 or 80 μM [γ-^{32}P]aATP (New England Nuclear or ICN, sp act. from 14.5 to 31 Ci/mmol) or [α-^{32}P]aATP (ICN, sp act. 30 Ci/mmol) in 50 mM Tris-HCl (pH 7.6) and 25 mM KCl for 25 min at 0 or 20 °C; initial experiments contained 1.0 mM $MgCl_2$, and later experiments included $MgCl_2$ at 5 mM (see text). Aliquots were either incubated in the dark (without UV irradiation) for 25 min at room temperature or were irradiated for 25 min with either an Aminco SPF-500 spectrofluorometer at 300-nm excitation and an 8-nm band-pass (296–304 nm) or a medium-wavelength UV light (Ultraviolet Products UVM 57, 520 μW/cm^2 at 302 nm and 15 cm). These procedures allow selective activation of the photoprobe without damage to protein (Koberstein et al., 1976). Since identical results were obtained in preliminary experiments, the latter procedure was selected for routine use.

For enzyme characterization, [γ-^{32}P]aATP was diluted with unlabeled aATP (Sigma) to a specific activity of 3×10^7 cpm/μmol, and time of incubation and concentration of aATP were varied. In other experiments, aATP was prephotolyzed by exposure to UV irradiation before incubation with NE suspensions (see Results).

To investigate the potential role of phosphorylation with [γ-^{32}P]aATP, NE suspensions were incubated (as above) with 500 μM [γ-^{35}S]ATP (New England Nuclear) for 10 min at room temperature; while phosphorylation of proteins occurs with this reagent, the resultant thiophosphorylated proteins are phosphatase resistant (Cassel & Glaser, 1982). In other experiments, NE preparations were incubated with 500 μM unlabeled γ-SATP (as above), harvested by centrifugation, and resuspended and photolabeled with [γ-^{32}P]aATP as described. Phosphorylation of NE proteins was examined after incubation of NE suspensions with 80 μM [γ-^{32}P]ATP for 10 min at room temperature.

Following photolabeling or preincubation experiments, mixtures were diluted 50-fold with TKM buffer (also containing 5 mM NaF and 5 mM ATP after phosphorylation experiments). NE were pelleted by centrifugation at 40000*g* for 15 min at 4 °C and resuspended in buffer and recentrifuged 2 times.

Labeled NE proteins were separated by electrophoresis in 7.0 or 10% polyacrylamide gels with 0.1% SDS (sodium dodecyl sulfate) (Laemmli, 1970) for 4 h at 80 V or 16 h at 25 V. Gels were stained with Coomassie blue, destained in 7% acetic acid, and dried onto backing sheets. Autoradiographs were made with Kodak X-omat-AR film after 8–72-h exposures at −70 °C. Gels and autoradiographs were scanned on an Ultroscan 2202 laser densitometer (LKB). Radioactivity was measured in bands cut from gels after homogenization in H_2O; these activities showed a good correlation between peak height of densitometer tracings of autoradiographs and radioactivity.

Results

The effect of irradiation on aATP was measured at room temperature for 25 min. Selective irradiation with 300-nm wavelength light resulted in spectral changes (Figure 1A) consistent with activation of the photoprobe, while irradiation of NE suspensions in the absence of aATP (see Figure 1B and legend) did not affect the activity. When incubated with NE suspensions in the dark (in mixtures containing 5 mM $MgCl_2$), hydrolysis of aATP occurred with a K_m of 0.225 mM and a

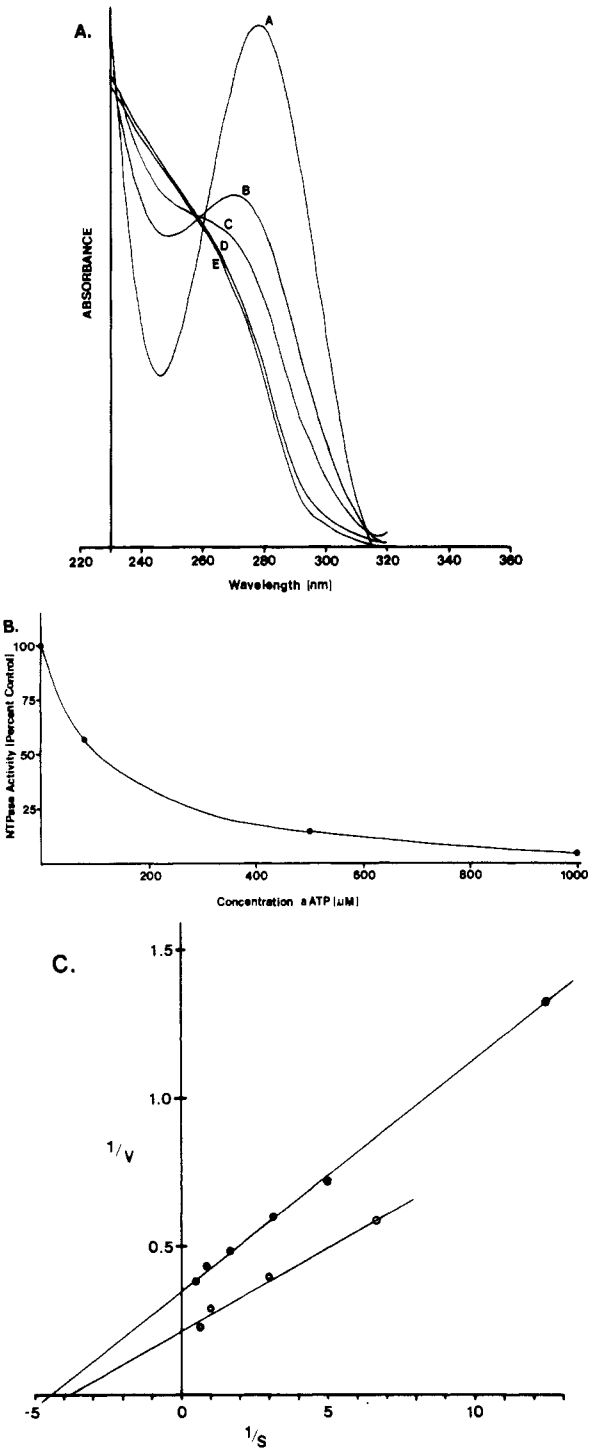

FIGURE 1: Characteristics of photoaffinity labeling with aATP. (A) Activation of aATP by UV irradiation. An 80 μM solution of aATP in TKM buffer was irradiated with 296–304-nm wavelength UV light with an Aminco SPF-500 spectrofluorometer in the excitation mode; at various times, the cuvette was withdrawn and the spectrum obtained in a Cary Model 118 spectrophotometer. An identical activation curve was obtained from irradiation with mid-wavelength UV light (UVP UVM-57, 520 μW/cm^2 at 302 nm and 15 cm). Curves A–E represent absorption spectra after 0, 5, 10, 20, or 30 min. (B) Inactivation of NE NTPase by aATP. NE suspensions were irradiated as in (A) in the presence of various concentrations of aATP. After 25 min, NE were obtained by centrifugation and resuspended and assayed for NTPase activity with [γ-^{32}P]ATP. Parallel experiments showed that inactivation was dependent upon UV irradiation. (C) Lineweaver–Burk graph of NE NTPase hydrolysis of aATP and ATP. NE suspensions were incubated with multiple concentrations of [γ-^{32}P]aATP or [γ-^{32}P]ATP. After incubation, release of γ-^{32}P was quantitated as described for ATP with activated charcoal (Clawson, et al., 1980a). Solid circles represent hydrolysis data for aATP; open circles represent those with ATP.

V_{max} of 2.83 μM h^{-1} (mg of protein)$^{-1}$, compared with values of 0.26 mM and 4.22 μM h^{-1} mg^{-1} with ATP as substrate (Figure 1C). These results suggest that aATP is an effective substrate for the NTPase. Quercetin, a potent inhibitor of NE protein kinase (Agutter et al., 1979a), did not inhibit the photolabeling at 5 mM $MgCl_2$ concentration, although some inhibition was noted with $MgCl_2$ at 1 mM (see below).

Preincubation of NE preparations with aATP, followed by recentrifugation, led to an irreversible reduction of NTPase activity upon subsequent reincubation with [^{32}P]ATP (Figure 1B). This reduction increased with time and aATP concentration and was dependent upon UV irradiation: irradiation of NE suspensions for 25 min with aATP at 80 μM led to a reduction of 57% compared to suspensions irradiated without aATP or incubated in the dark (which showed hydrolysis similar to control suspensions), while aATP at 500 μM or 1 mM reduced the NTPase by 85–95%. When ATP or GTP (at 1 or 5 mM) was included in the suspensions with aATP, they protected against the UV-dependent inactivation, with subsequent reincubation showing 100% of the control NTPase activity. These data demonstrate a UV-dependent inactivation of NTPase activity by photoaffinity label.

Under the conditions employed, ATP or GTP was able to competitively inhibit the UV-dependent incorporation of label from [γ-^{32}P]aATP; for instance, inclusion of ATP at a ratio of 1.25:1 (ATP to aATP) decreased incorporation by 20% and at 62.5 by nearly 100%. Similar results were obtained when GTP was competed with aATP. In contrast, UTP was only half as effective, decreasing incorporation by 10% at a ratio of 1.25.

In other experiments, [γ-^{32}P]aATP was photolyzed prior to incubation with NE preparations; this significantly reduced photolabeling and eliminated UV dependence; ATP and GTP did not effectively compete the labeling; and the specificity of labeling was altered (see below). These data indicate that the photolabel interacts with the same activity responsible for hydrolysis of NTP's.

We next attempted to label and identify the protein responsible for this activity. In initial experiments to determine specificity of labeling, NE proteins were photoaffinity labeled with [γ-^{32}P]aATP in 1 mM $MgCl_2$ and were examined by polyacrylamide gel electrophoresis and autoradiography. In the absence of UV irradiation, only diffuse labeling of a 58-kDa band occurred. Following UV irradiation, a second major band with a mass of 46 kDa (containing 40% of the incorporated label) was found (see inset to Figure 2). Under the conditions employed, quercetin resulted in a partial reduction of labeling, suggesting that some phosphorylation may have occurred. These results may reflect the fact that NE protein kinase activity is maximal at 1 mm $MgCl_2$, whereas NE NTPase has optimal activity at a higher magnesium concentration (Ahmed & Steer, 1982).

Subsequent experiments were performed at 5 mM $MgCl_2$, which supports maximal NTPase activity (data not shown). These studies resulted in photoaffinity labeling that was more than 80% dependent upon UV irradiation and that showed a highly selective labeling pattern (Figures 2 and 3). In preparations incubated in the dark, the heaviest incorporation of label was found in a diffuse band at 58 kDa with [γ-^{32}P]aATP at 8 or 80 μM (Figures 2B and 3C; in some instances, this band could be seen to consist of two or more components). This presumably represents use of γ-^{32}P in phosphorylation reactions. In this regard, the 58-kDa band was also one of the major NE bands phosphorylated in mixtures containing 80 μM [γ-^{32}P]ATP (Figure 3D).

Following UV irradiation, a major 46-kDa band was labeled (Figures 2C and 3B). With 8 μM [γ-^{32}P]aATP, incorporation of label into the 46-kDa band was equivalent to that in the 58-kDa band, and significant incorporation was also observed in four larger bands at 180, 98, 91, and 74 kDa. With 80 μM [γ-^{32}P]aATP, the 46-kDa band was the predominant band detected on autoradiographs, containing more than 60% of total incorporated activity (see Figure 3B). At either concentration, labeling of the 46-kDa band was greatly reduced or eliminated in the absence of UV irradiation. This band represents a minor protein component and, hence, has a specific activity 7–20 times that of the diffuse 58-kDa band. Photolabeling of a number of minor components was also observed at both reagent concentrations with indicated molecular weights of 180, 98, 91, 85, 79, 74, 69, and 51 kDa (see Figures 2 and 3 and Discussion).

Some notable differences exist between photolabeling results obtained with 8 and 80 μM [γ-^{32}P]aATP. At the lower concentration (8 μM), heavier labeling of the higher molecular weight bands occurred, and the 46-kDa band was equal in intensity to the 58-kDa band (Figure 2C). At the higher concentration (80 μM), labeling of the 46-kDa band was greatly increased, and that of the higher molecular weight bands was proportionally reduced (Figure 3B). It is also apparent that much heavier labeling of the higher molecular weight polypeptides occurred in the dark at 8 μM, compared with 80 μM (Figures 2B and 3C).

For comparison, we examined NE preparations for phosphorylation of proteins after incubation with 80 μM [γ-^{32}P]ATP. A distinctly different pattern of labeling was observed. The major bands phosphorylated had molecular weights of 69 and 58 kDa, and there was no detectable labeling of the 46-kDa band (Figure 3D).

When prephotolyzed [^{32}P]aATP was employed in photolabeling experiments, no labeling of the 46-kDa band occurred (Figure 2D). A number of minor bands were also photolabeled (which closely match those phosphorylated with [γ-^{32}P]ATP or aATP), and major labeling of the diffuse 58-kDa band again was observed.

In an attempt to further define the role of phosphorylation under these conditions, NE preparations were preincubated with γ-SATP and harvested by centrifugation. Control experiments with γ-^{35}SATP demonstrated that phosphorylation occurred with this reagent; further, the thiophosphorylated proteins are phosphatase resistant (Cassels & Glaser, 1982). NE preparations, previously phosphorylated with γ-SATP, were then photolabeled with [γ-^{32}P]aATP. When reactions were carried out in the dark, no discernible bands were identified. Following UV irradiation, labeling of the 58-kDa band was dramatically reduced, and the only discernible band was found at 46 kDa (Figure 2A; a high level of background labeling occurred at each end of the gel, and overexposure was necessary to demonstrate this band).

Our results suggest that photolabeling of polypeptides under the conditions employed does not reflect use of γ-^{32}P in phosphorylation of NE proteins. To directly confirm this, we examined NE photolabeled with [α-^{32}P]aATP: With this reagent, we observed a pattern of photolabeling very similar to that obtained with [γ-^{32}P]aATP with a slightly reduced percentage of radioactivity associated with the 46-kDa band (see Figures 4 and 5). In contrast to our results with [γ-^{32}P]aATP, however, no labeled protein bands were detected on gels when labeling experiments with [α-^{32}P]aATP were conducted in the dark (Figure 5A).

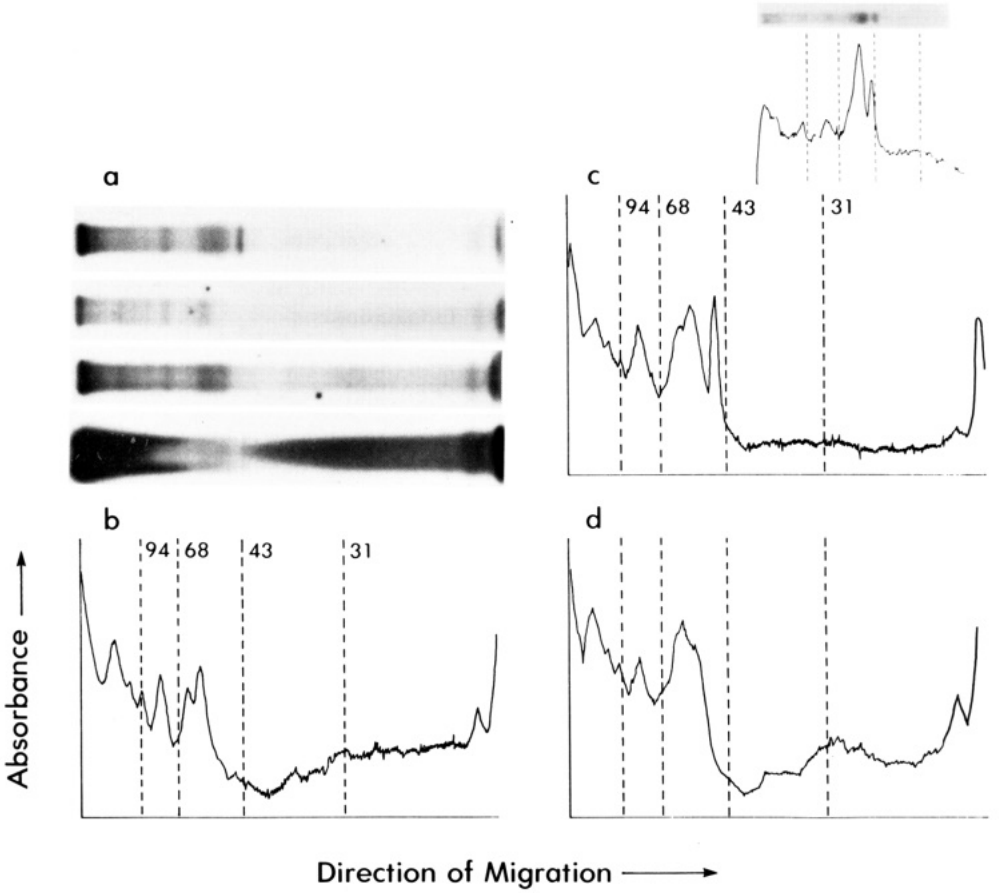

FIGURE 2: Photoaffinity labeling of NE with 8 μM [γ-^{32}P]aATP or prephotolyzed photoprobe. NE were resuspended in 8 μM [γ-^{32}P]aATP in mixtures containing 5 mM $MgCl_2$. Aliquots were either irradiated with 300-nm UV light during incubation at room temperature for 25 min or were incubated in the dark. After incubation, NE were obtained by centrifugation 3 times and were electrophoresed on 10% polyacrylamide slab gels (10 × 15 × 0.75 cm) as described by Laemmli (1970) for 4 h at a constant voltage of 80 V. Gels were then stained with Coomassie blue dye, destained in 7% acetic acid, and dried on Bio-Rad backing sheets. Autoradiographs were made by exposure for 8–72 h at −70 °C on Kodak X-omat-AR film. Gels and autoradiographs were then scanned with a LKB 2202 ultroscan laser densitometer and compared with standards run on the same gels. Experiments in which bands were cut from the gels and radioactivity was measured after homogenization in H_2O showed a good correlation between peak height on densitometer tracings of autoradiographs and radioactivity in gels under the scanning conditions utilized (not shown). (a) Autoradiographs of gels of NE preparations photolabeled with (second from top) [γ-^{32}P]aATP in the dark, (top) [γ-^{32}P]aATP with UV irradiation, (second from bottom) prephotolyzed [γ-^{32}P]aATP with UV irradiation, and (bottom) thiophosphorylated NE preparation photolabeled with [γ-^{32}P]aATP with UV irradiation. Parts B–D represent densitometric tracings of autoradiographs after polyacrylamide gel electrophoresis of NE preparations photolabeled (B) in the dark, (C) with UV irradiation, (D) with prephotolyzed [γ-^{32}P]aATP and UV irradiation. Inset (upper right) shows autoradiograph and densitometric tracing of NE preparation photolabeled with 80 μM [γ-^{32}P]aATP in an incubation mixture containing 1 mM $MgCl_2$. Dotted lines, left to right, represent position of phosphorylase B (94 kDa), bovine albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (31 kDa). The autoradiographs in (A) are aligned with these markers. A variable amount of label was associated with the dye front and did not affect the pattern of photoaffinity labeling.

Since the NTPase activity implicated in RNA transport is modulated by RNA (Agutter et al., 1979; Clawson et al., 1980a), we examined the effects of removing RNA from NE preparations and of readding exogenous RNA to such preparations. NE preparations subjected to the additional RNase/DNase digestion showed dry weight contents of 1.5–2.0% RNA and 3–4% DNA, compared with 8–9% RNA and 3–5% DNA in our standard preparations, and the NTPase activity in such preparations was decreased by 30–40% under standard assay conditions. Photolabeling of these suspensions with 8 or 80 μM [α-^{32}P]aATP resulted in major labeling of only the 58-kDa band (Figures 4 and 5) even though the 46-kDa band was still present (the band appears on Coomassie blue stained gels). Yeast RNA at 200 μg/mL was then added to these NE suspensions: this concentration results in maximal stimulation of NE NTPase activity to levels comparable to undigested preparations. This amount of RNA represents addition of about 5 times the endogenous quantity of RNA removed by RNase/DNase digestion. Photolabeling of the 46-kDa band was again seen (Figure 4D). When radioactivity was assessed in bands removed from gels, we found that 35–45% of the incorporated radioactivity was present in the 46-kDa band with RNA included in the photolabeling experiments, compared with no significant labeling without added RNA.

In other experiments, varying concentrations of yeast RNA were added to DNase/RNase-treated NE suspensions, and photolabeling with [α-^{32}P]aATP was performed with UV irradiation. NE proteins were separated by polyacrylamide gel electrophoresis, and the label in the 58- and 46-kDa bands was assessed (by peak height on densitometric tracings, later checked by assessment of radioactivity). The results show no change in labeling of the 58-kDa band (Figure 5B), whereas a modulation of photolabeling of the 46-kDa band was observed, which was maximal at 200 μg/mL RNA addition (Figure 5C). Paralleling this modulation, we measured an increase in NTPase at 200 μg/mL (back to the control level), but we do not find a significant increase with 50, 100, or 500 μg/mL yeast RNA added to DNase/RNase-pretreated preparations (see legend to Figure 5).

We also found an increasing incorporation of label into the two smaller bands at 35 and 30 kDa with 200 and 500 μg/mL

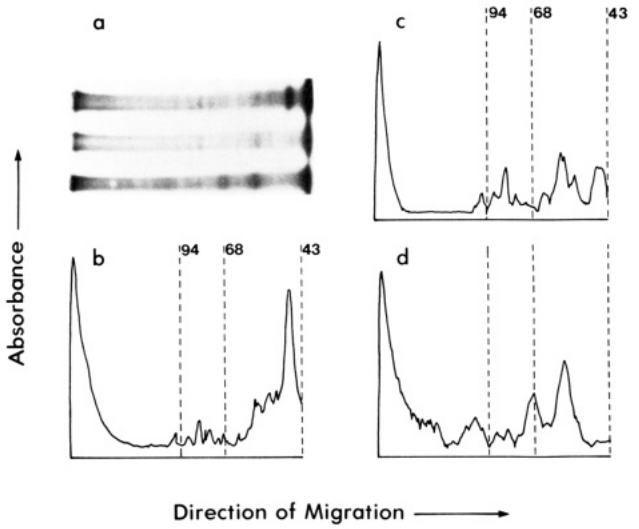

FIGURE 3: Photoaffinity labeling of NE with 80 μM [γ-^{32}P]aATP or [γ-^{32}P]ATP. NE were resuspended in 80 μM [γ-^{32}P]aATP or 80 μM [γ-^{32}P]ATP in mixtures containing 5 mM $MgCl_2$; incubation for photoaffinity labeling was as in Figure 1, and for phosphorylation reactions with [γ-^{32}P]ATP, incubations were for 10 min at room temperature. NE were then obtained by sedimentation 2 times and were electrophoresed in 7% polyacrylamide slabs gels for 16 h at a constant voltage of 25 V, which allowed better separation of the pertinent bands. Gels were then processed as described. (A) Autoradiographs of gels showing NE proteins after photoaffinity labeling with UV irradiation (top) or in the dark (middle) or after phosphorylation with [γ-^{32}P]ATP (bottom). Parts B–D show densitometric tracings of autoradiographs of NE proteins after (B) photoaffinity labeling with UV irradiation in suspensions containing 80 μM [γ-^{32}P]aATP, (C) photoaffinity labeling in the dark (without UV irradiation), and (D) labeling with [γ-^{32}P]ATP for 10 min. Dotted lines (left to right) represent the positions of 94-, 68-, and 43-kDa markers; the autoradiographs in (A) are aligned with these markers. The molecular weight of the major photoaffinity labeled band is approximately 46 kDa, which corresponds to a quantitatively minor protein band. The minor photoaffinity-labeled bands [in both (B) and (C)] indicate molecular masses of 98, 91, 85, 79, 74, 69, 58, 51, and 49 kDa. The major bands phosphorylated with [γ-^{32}P]ATP indicate molecular masses of 69 and 58 kDa with much fainter bands at 180, 105, 98, 85, 79, 72 kDa.

RNA (see Figure 4D and inset to Figure 5). The greatly increased labeling of these bands at 500 μg/mL RNA may reflect binding of ATP (or aATP) and may explain the lack of increase in NE NTPase activity at this higher concentration of RNA.

Discussion

Our results demonstrate that aATP is an effective substrate for NE NTPase and that it results in a UV, time, and concentration dependent irreversible inactivation of NTPase. The reaction of aATP with the NTPase is competitively blocked by ATP or GTP. This photoprobe labels two major NE protein bands of M_r 58 000 and 46 000. We suggest that the 46-kDa band represents the NTPase implicated in RNA transport for the following reasons: (1) photolabeling of the 46-kDa band is totally dependent upon UV irradiation, and no phosphorylation of this band occurs with [γ-^{32}P]ATP or [γ-^{32}P]aATP; (2) under optimal labeling conditions, it is the major band labeled, while labeling of the broad 58-kDa band also involves phosphorylation (see below); (3) the specific activity of this band is 7–20 times that of the 58-kDa band; (4) the extent of its labeling in 8 or 80 μM aATP suggests it is a relatively low-affinity size, as is the NTPase; (5) removal/addition of RNA specifically modulates photolabeling of this band, paralleling modulation of NE NTPase activity. This latter point is particularly important since it suggests that the

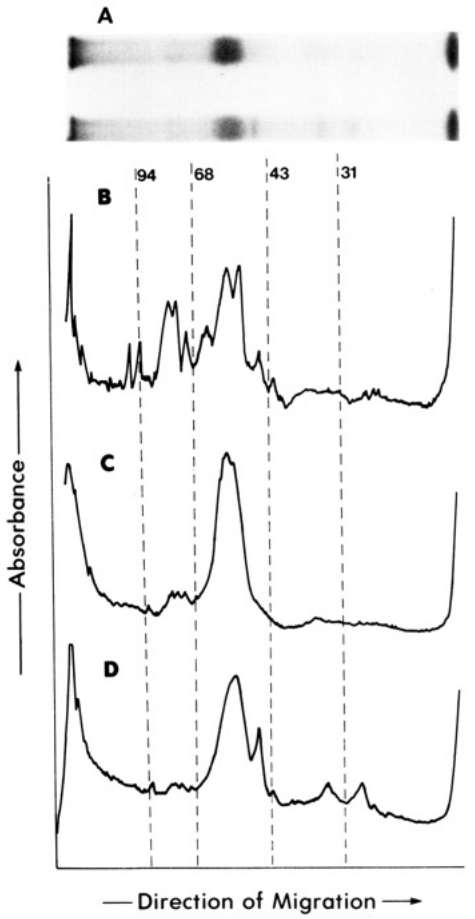

FIGURE 4: Photoaffinity labeling of NE polypeptides with [α-^{32}P]-aATP: modulation by RNA. RNase/DNase-treated NE preparations were resuspended in TKM buffer with 8 or 80 μM [α-^{32}P]aATP (ICN). A portion of each preparation was incubated in the dark for 25 min at room temperature; other portions, with or without addition of 200 μg/mL yeast RNA, were exposed to long-wavelength UV light under the same conditions. The photoaffinity-labeled NE preparations were harvested by centrifugation and were resuspended and rinsed twice. The photolabeling was totally dependent on UV irradiation. The preparations were electrophoresed in 10% polyacrylamide gels. The gels were dried onto backing sheets and autoradiographs were developed after exposure to dried gels for 24–48 h at −70 °C. Gels and autoradiographs were scanned with an Ultroscan 2202 laser densitometer (LKB). Dotted lines (left to right) represent positions of phosphorylase B (94 kDa), bovine albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (31 kDa). (A) Autoradiographs of NE proteins after photoaffinity labeling with 8 μM [α-^{32}P]aATP. The top track represents photolabeling in the absence of RNA, while the bottom represents that obtained in the presence of 200 μg/mL yeast RNA. (B) Densitometric tracing of Coomassie blue stained polyacrylamide gel. The peaks at 79, 74, and 69 kDa appear to correspond to lamins A, B, and C, respectively. The largest peaks indicate molecular masses of 58 and 55 kDa. Portions of this preparation were photolabeled in the absence (C) or presence (D) of 200 μg/mL yeast RNA. (C) Densitometric tracing of autoradiograph of electrophoresed proteins photolabeled without added RNA. The major peak indicates a molecular mass of 58 kDa. The minor peaks of 91, 79, 74, and 69 kDa were also photolabeled in NE preparations not subjected to DNase/RNase treatment and probably represent pore-complex components (see Discussion). (D) Densitometric tracing of autoradiograph of electrophoresed proteins photolabeled in the presence of added RNA. The largest peak indicates a molecular mass of 58 kDa, with a major peak now evident at 46 kDa. In experiments where 58-, 55-, and 46-kDa bands were cut from gels, allowed to swell in H_2O, homogenized, and radioactivity assessed, we found that 35–45% of the total radioactivity was present in the 46-kDa band. The minor peaks of 91, 79, 74, and 69 kDa are again identified [as in (C)]. Two minor peaks are identified near 31 kDa (see Figure 5).

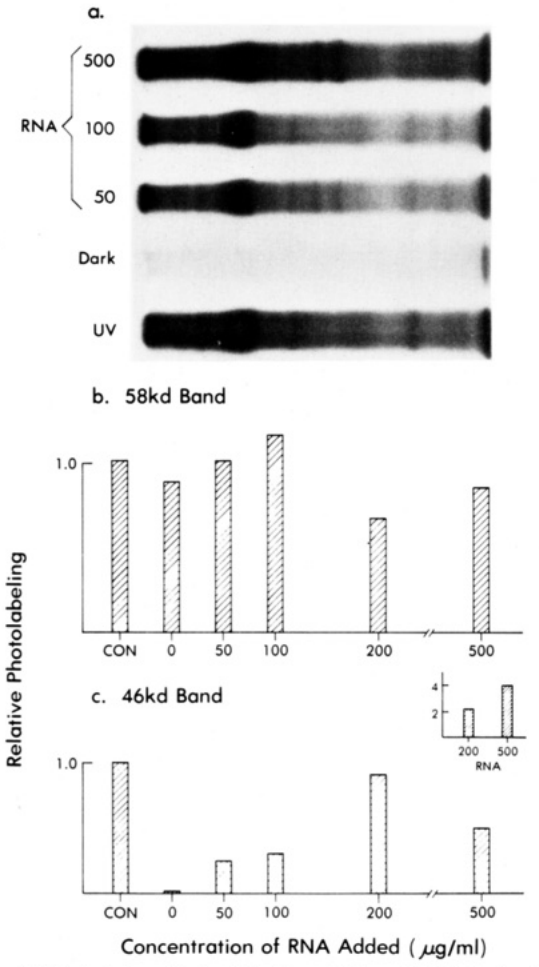

FIGURE 5: Modulation of photolabeling of 46- and 58-kDa bands by RNA. Control or RNase/DNase-treated NE preparations were resuspended in TKM buffer with 8 μM [α-^{32}P]aATP. Half of the control preparation was incubated in the dark for 25 min at room temperature and half was UV irradiated. Yeast RNA at 0, 50, 100, 200, or 500 μg/mL was added to RNase/DNase-treated NE preparations, and these were incubated for 25 min at room temperature with UV irradiation. Following photolabeling, NE preparations were handled as described (legend to Figure 4) with exposure of autoradiographs for 8–72 h. (A) Autoradiographs of control NE preparations labeled with or without UV irradiation (as labeled) and of RNase/DNase-treated preparations photolabeled in the presence of 50, 100, or 500 μg/mL yeast RNA (examples of labeling in the presence of 0 and 200 μg/mL yeast RNA are shown in Figure 4). Densitometric tracings were made of the autoradiographs under identical scanning conditions, and peak heights of the 58- (B) and 46-kDa (C) bands were carefully quantitated. With yeast RNA at 200 or 500 μg/mL, significant labeling of two smaller molecular mass peaks (~35 and ~30 kDa) occurred. The inset of (C) shows the combined peak heights of these two peaks plus the 46-kDa peak. Assessment of NTPase activity showed increases of 10–20% with 50, 100, and 500 μg/mL RNA added to DNase/RNase-digested preparations (as compared to no addition), but these differences were not significant. However, NTPase activity was significantly increased (to control levels) with addition of 200 μg/mL RNA (see text).

46-kDa band does not represent actin. An intriguing possibility is that the 46-kDa protein represents that involved in binding of hnRNA to the nuclear scaffolding (van Eckelen & van Vendrooij, 1981).

With regard to the 58-kDa band, observed are the following: (1) it is phosphorylated with [γ-^{32}P]aATP or [γ-^{32}P]ATP (see Figures 2B and 3C,D); (2) its labeling is proportionally increased in the dark (Figure 3C), in lower concentrations of aATP (compare Figures 2C and 3B), and under conditions favoring protein kinase activity (compare Figure 2 inset and Figure 3B); (3) its photolabeling is eliminated when thiophosphorylated NE preparations are employed (Figure 2A); (4) its photolabeling is not modulated by RNA concentration (Figure 5). This band is also photolabeled in a UV-dependent manner with [α-^{32}P]aATP (see text and Figure 4B,C), and it seems clear from quantitative considerations that it also photolabeled with [γ-^{32}P]aATP (since 80% of the labeling is UV dependent). Since proteins of this molecular mass are thought to be prominent components of nuclear pore complexes and since nuclear pores provide aqueous pathways and are positively charged at physiologic pH (Feldherr, 1974), we suggest that the 58-kDa band is also photolabeled as a result of nucleotides localizing to nuclear pores on the basis of charge attraction. This supposition would explain why photolabeling of this band is eliminated when proteins are thiophosphorylated, since thiophosphorylation would presumably eliminate any charge attraction. However, we cannot rule out a minor role for the diffuse 58-kDa band as an NTPase.

While the significance of NE phosphorylation is unknown, phosphorylation/dephosphorylation processes appear to participate in regulation of many metabolic and membrane activities (Allfrey et al., 1973; Rubin & Rosen, 1975; Greengard, 1978; Krebs & Beavo, 1979; Cohen, 1982) including regulation of NE NTPase activity, where dephosphorylation has been hypothesized to be rate limiting and to furnish the active ATPase(s) (MacDonald & Agutter, 1980). It is therefore pertinent that the 46-kDa band was not phosphorylated by endogenous kinases.

The other minor photolabeled bands indicate molecular masses of approximately 180, 98, 91, 85, 79, 74, 69, and 51 kDa. The importance of these proteins as NTPases is unknown. The 85-kDa band corresponds to a minor protein band. The 79-, 74-, and 69-kDa bands correspond to lamins A–C (respectively) in our hands. Proteins of the other approximate molecular weights are thought to be associated with the nuclear pore complexes (Richardson & Maddy, 1980a), and thus, these components may also have been labeled by aATP, which was lured to the microenvironment of the pore complex by charge attraction. A nonspecific labeling (particularly of the 58-kDa band) may also in part reflect the fact that the NE NTPase is a relatively low-affinity site.

One of the minor photoaffinity-labeled bands indicates an approximate M_r of 180000, similar to that reported by Berrios et al. (1983) for a 174-kDa polypeptide; they suggest this polypeptide band as a likely candidate for the matrix-pore complex-lamina fraction ATPase/dATPase from *Drosophila*. The properties of this matrix activity differ from those of the NTPase associated with RNA transport and appear to differ in substrate specificity from rat liver nuclear matrix NTPase (Clawson & Smuckler, 1982; Berrios et al., 1983). Interestingly, these investigators also reported heavy labeling of two broad autoradiographic bands near 55 kDa with a [α-^{32}P]GTP substrate. The considerably higher K_m (1 mM) reported for the GTPase (Berrios et al., 1983) may have precluded significant labeling at the 0.1 μM concentrations of ATP or dATP in the their labeling experiments, and their use of 0.1 mM $MgCl_2$ may also have reduced NTPase binding of substrate, since it appears that selective and reproducible labeling requires higher $MgCl_2$ concentrations. Further, photolabeling of the 180-kDa band we obtained was proportionally enriched in the lower concentration of aATP tested, which suggests that this site has a higher affinity than the 46-kDa NTPase. We also note that GTP is as effective a substrate as ATP in RNA transport and NE NTPase assays with rat liver preparations and that GTP competed with aATP in our photolabeling experiments.

Our results suggest that the inhibition of RNA transport and NTPase activity by lamin B antibodies observed by Baglia & Maul (1983) derives from a mechanism other than direct binding of antibodies to the major NTPase itself. In an attempt to directly demonstrate NTPase activity, NE proteins were separated in 7% polyacrylamide gels. SDS was eluted with 2-propanol-containing buffer (Blank et al., 1982), gels were incubated in modified Wachstein–Meisel medium (Wachstein & Meisel, 1957) for 2 h at room temperature and rinsed in H_2O, and lead phosphate precipitate was visualized by staining with 1% ammonium sulfide. This procedure was not successful, a result for which we have no ready explanation at present. However, further separations should allow purification of the active enzyme and the development of specific antibodies.

Acknowledgments

We thank David A. Geller for fine editorial assistance.

Registry No. aATP, 53696-59-6; NTPase, 9075-51-8; ATP, 56-65-5; GTP, 86-01-1.

References

Agutter, P., Cockrill, J., Lavine, J., McCaldin, B., & Sim, R. (1979a) *Biochem. J. 181*, 647.

Agutter, P., McCaldin, B., & McArdle, H. (1979b) *Biochem. J. 182*, 811.

Ahmed, K., & Steer, R. (1982) *Wistar Symp. Ser. 2*, 31.

Allfrey, V., Inoue, A., Karn, J., Johnson, E., & Vidali, G. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38*, 785.

Baglia, F., & Maul, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2285.

Berezney, R. (1979) *Cell Nucl. 7*, 413.

Berrios, M., Blobel, G., & Fisher, P. (1983) *J. Biol. Chem. 258*, 4548.

Blank, A., Sugiyama, R., & Dekker, C. (1982) *Anal. Biochem. 120*, 267.

Cassel, D., & Glaser, L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2231.

Clawson, G., & Smuckler, E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5400.

Clawson, G., & Smuckler, E. (1982) *Biochem. Biophys. Res. Commun. 108*, 1131.

Clawson, G., Koplitz, M., Castler-Schechter, B., & Smuckler, E. (1978) *Biochemistry 17*, 3747.

Clawson, G., James, J., Woo, C., Friend, D., Moody, D., & Smuckler, E. (1980a) *Biochemistry 19*, 2748.

Clawson, G., Koplitz, M., Moody, D., & Smuckler, E. (1980b) *Cancer Res. 40*, 75.

Cohen, P. (1982) *Nature (London) 296*, 613.

Comings, D., & Okada, T. (1976) *Exp. Cell Res. 103*, 341.

Dwyer, N., & Blobel, G. (1976) *J. Cell Biol. 70*, 581.

Feldherr, C. (1974) *Exp. Cell Res. 85*, 271.

Franke, W. (1974) *Int. Rev. Cytol. 4*, 71.

Gerace, L., Blum, A., & Blobel, G. (1978) *J. Cell. Biol. 79*, 546.

Greengard, P. (1978) *Science (Washington, D.C.) 199*, 146.

Haley, B., & Hoffman, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3367.

Hollemans, M., Runswick, M., Fearnley, I., & Walker, J. (1983) *J. Biol. Chem. 258*, 9307.

Ishikawa, K., Kuroda, C., & Ogata, K. (1969) *Biochim. Biophys. Acta 179*, 316.

Jacobs, H., & Birnie, G. (1982) *Eur. J. Biochem. 121*, 597.

Knowles, J. (1972) *Acc. Chem. Res. 5*, 155.

Koberstein, R., Cobianchi, L., & Sund, H. (1976) *FEBS Lett. 64*, 176.

Krebs, E., & Beavo, J. (1979) *Annu. Rev. Biochem. 48*, 923.

Krohne, G., Franke, W., & Scheer, U. (1978) *Exp. Cell Res. 116*, 85.

Laemmli, U. (1970) *Nature (London) 227*, 680.

Lam, K., & Kasper, C. (1979) *J. Biol. Chem. 254*, 11713.

Maul, G. (1977) *Int. Rev. Cytol., Suppl. 6*, 75.

McDonald, J., & Agutter, P. (1980) *FEBS Lett. 116*, 145.

Murty, C., Hornseth, R., Verney, E., & Sidransky, H. (1983) *Lab. Invest. 48*, 256.

Purrello, F., Vigneri, R., Clawson, G., & Goldfine, I. (1982) *Science (Washington, D.C.) 216*, 1005.

Racevskis, J., & Webb, T. (1974) *Eur. J. Biochem. 49*, 93.

Raskas, H. (1971) *Nature (London), New Biol. 233*, 134.

Richardson, J., & Maddy, A. (1980a) *J. Cell Sci. 43*, 269.

Richardson, J., & Maddy, A. (1980b) *J. Cell Sci. 43*, 253.

Rubin, C., & Rosen, O. (1975) *Annu. Rev. Biochem. 44*, 831.

Shaper, J., Pardoll, D., Kaufman, S., Barrack, E., Vogelstein, B., & Coffey, D. (1979) *Adv. Enzyme Regul. 17*, 213.

Shelton, K., Guthrie, V., & Cochran, D. (1980) *Biochem. Biophys. Res. Commun. 93*, 867.

Shia, M., & Pilch, P. (1983) *Biochemistry 22*, 717.

Unwin, P., & Milligan, R. (1982) *J. Cell Biol. 93*, 63.

van Eckelen, C., & van Vendrooij, W. (1981) *J. Cell Biol. 88*, 554.

Wachstein, M., & Meisel, E. (1957) *Am. J. Clin. Pathol. 27*, 13.

Wagenvoord, R., van der Kraan, I., & Kemp, A. (1977) *Biochim. Biophys. Acta 460*, 17.