Characterization of a ribonuclease-sensitive nucleoside triphosphatase activity from HeLa nuclei

Lislott V. RICHARDSON and John P. RICHARDSON

Department of Chemistry, Indiana University, Bloomington, IN 47405, U.S.A.

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Approximately one-third of the total ATP-hydrolysis activity in isolated HeLa nuclei is sensitive to RNAase (ribonuclease). This activity is selectively extracted with pulse-labelled RNA. In the extracts it co-sediments with various particles with sedimentation coefficients from 10S to 50S, but especially with 24S and 40S particles. ATP hydrolysis by the isolated particles was inhibited extensively (>80%) by RNAase A, heparin and 0.2M-NaCl. The activity of RNAase-treated particles was recovered when poly(A) was added, but not when DNA was added. The isolated particles exhibited RNAase-sensitive hydrolysis activities for dATP, GTP, CTP and UTP as well as for ATP, and the UTPase activity in the extracts showed nearly the same sedimentation distribution as the ATPase activity. When samples of isolated particles were irradiated with u.v. light in the presence of [α - 32 P]ATP, a 39kDa polypeptide with a broad distribution from 10S to 50S like that of the ATPase and a 55kDa polypeptide with a sharp distribution at 24S were photolabelled. Taken together, the data suggest that ATP-hydrolysis activity found in nuclear ribonucleo-protein subfractions appears to be the result of one or two RNA-dependent NTPases that are normally associated with endogenous RNA in a wide variety of particles.

Nuclei from animal cells contain specific ATP phosphohydrolases (ATPases) that have not been thoroughly characterized (Siebert & Humphrey, 1965). Since several proteins involved with DNA and RNA metabolism have associated ATPase activities, many of the specific nuclear ATPases are likely to have functional roles in DNA replication and repair or in RNA synthesis and maturation.

Most of the known examples of DNA- or RNA-dependent ATPases are from bacterial sources. *Escherichia coli* has as many as 12 distinct enzymes that catalyse the hydrolysis of ATP in the presence of DNA (Kornberg, 1980). However, the only known *E. coli* RNA-dependent ATPase is RNA-synthesis termination factor ρ (Lowery-Goldhammer & Richardson, 1974; Finger & Richardson, 1981), a protein that couples NTP hydrolysis to the termination and release of RNA molecules (Richardson & Conaway, 1980).

RNA-dependent ATPases have been identified

Abbreviations used: NTPase, nucleoside triphosphate phosphohydrolase; RNAase, ribonuclease; DNAase, deoxyribonuclease; PMSF, phenylmethanesulphonyl fluoride; RNP, ribonucleoprotein.

in a number of eukaryotic organisms (Agutter *et al.*, 1977; Belhadj *et al.*, 1980; Chumakov *et al.*, 1982; Blanchard & Richardson, 1983), but none of these enzymes has been characterized as extensively as ρ factor, and little or nothing is known about their functions. In this paper we measure the amount of RNAase-sensitive ATPase activity associated with nuclei from HeLa cells and analyse its subnuclear localization. A method for preparation of a nuclear subfraction that contains a significant fraction of the RNAase-sensitive ATPase activity with very little of the other nuclear ATPases is presented, and properties of the ATPase activity in that nuclear subfraction are described.

Experimental

Materials

Unlabelled ribonucleoside triphosphates were purchased from Boehringer Mannheim Corp.; dATP and poly(A) were from P-L Biochemicals. [α - 32 P]ATP, $H_3^{32}PO_4$, [5- 3 H]uridine and [methyl- 3 H]thymidine were purchased from ICN Pharmaceuticals. [γ - 32 P]ATP and [γ - 32 P]UTP were prepared by the method of Calva *et al.* (1980). Calf

thymus DNA, RNAase A and DNAase I (code DPRF) were from Worthington. Heparin, ouabain, *N*-ethylmaleimide, PMSF and quercetin were from Sigma. Efrapeptin was provided by Eli Lilly Corp.

Culture labelling and fractionation of HeLa cells

HeLa cells (strain S-3, obtained from the M.I.T. Cell Culture Center) were grown in suspension culture at 37°C in RPMI 1640 synthetic medium (Grand Island Biological Co.) with 5% (v/v) horse serum. Cell density was maintained at approx. 4×10^5 cells/ml. To label heterogeneous nuclear RNA, cells were pelleted by centrifugation at 500g for 2min, resuspended at a concentration of 3×10^6 cells/ml, treated for 30min at 37°C with 0.04 µg of actinomycin D/ml and then incubated with 1 µCi of [5-³H]uridine/ml for 30min at 37°C with gentle shaking. To label DNA, cells were incubated for 24h with 1.5 µCi of [methyl-³H]thymidine/ml.

For the preparation of nuclei and nuclear fractions, cells were rapidly chilled, pelleted by centrifugation at 500g for 2min, and resuspended at a density of 2×10^7 cells/ml in RSB buffer (10mM - NaCl / 1.5mM - MgCl₂ / 10mM - Tris / HCl, pH 7.4). The cells were lysed by homogenization at 4°C in a Dounce homogenizer with the B pestle. After eight strokes, Nonidet P-40 was added to 0.5% and the suspension was treated with five more strokes. Nuclei were pelleted by centrifugation at 500g for 2min, suspended in 10 times their packed volume of RSB and pelleted again by centrifugation. This procedure was repeated until the supernatant was clear (from two to five washes). The pellet of washed nuclei was suspended at a density of 2×10^7 nuclei/ml in a solution of RSB in 50% (v/v) glycerol. This nuclear suspension was stored at -20°C.

RNP particles were extracted from intact HeLa nuclei by a method based on that described by Samarina *et al.* (1968). For a typical preparation, 8.5×10^7 nuclei were suspended in 1ml of 50mM-Na₃EDTA (pH 8.0) with 25 µg of PMSF/ml, treated for 5min at 0°C, pelleted by centrifugation at 700g for 2min, and resuspended in 1ml of 0.1M-NaCl/1mM-MgCl₂/10mM-Tris/HCl (pH 8.5) containing 25 µg of PMSF/ml. This solution was incubated for 2h at 37°C. The intact nuclei were removed by centrifugation at 700g for 5min. The supernatant solution is the crude RNP fraction.

Sucrose-gradient zone centrifugation of nuclear RNP fraction

Samples of 1.0ml of crude RNP fraction ($A_{260} = 17$) were layered on 14.3ml linear 15–30% (w/v) sucrose gradients containing 0.1M-NaCl, 1mM-MgCl₂, 10mM-Tris/HCl (pH 7.4) and 25 µg of PMSF/ml. The particles were separated by centri-

fugation for 7h at 40000 rev./min at 4°C in a Beckman SW41 rotor. Gradients were fractionated from the top with an ISCO density-gradient fractionator. The distribution of u.v.-absorbing material was monitored with an ISCO UA5 absorbance monitor at 254nm. Fractions (0.62ml) were collected and used for assays or pooled for further experiments. For studies of the general characterization of the ATPase associated with the particles, fractions containing particles with sedimentation coefficients from 14S to 50S were pooled, mixed with an equal volume of gradient buffer without sucrose, and subjected to centrifugation at 37000 rev./min for 14h in a Beckman 50Ti rotor. Each pellet from 1.0ml of extract was resuspended in 0.25ml of 0.1M-NaCl/1mM-MgCl₂/10mM-Tris/HCl (pH 7.4), and portions of the final solution were stored at -80°C.

NTPase assays

The standard reaction mixture for measuring hydrolysis of ATP contained 0.05M-Tris/HCl, pH 7.4, 1mM-MgCl₂, 1mM-ATP and 15 µg of poly(A)/ml. Hydrolysis was measured either by the release of [³²P]P_i from [γ-³²P]ATP as described by Blanchard & Richardson (1983), or by the formation of [³²P]ADP from [α-³²P]ATP as described by Arai *et al.* (1981). Incubations were for 10min at 37°C unless indicated otherwise. One unit is equal to 1nmol of ATP hydrolysed/min.

The hydrolyses of dATP, GTP, CTP and UTP were measured by a modification (Lowery & Richardson, 1977) of the colorimetric assay for P_i developed by Wahler & Wollenberger (1958).

Assays for [³H]RNA and [³H]DNA

To determine acid-insoluble radioactivity, a sample treated with 6% (w/v) trichloroacetic acid was collected on a 2.5cm Whatman GF/C glass-fibre filter, the filter was washed with 3 × 1ml of 2% trichloroacetic acid, and the damp filter was digested with 0.5ml of NCS fluid (Amersham/Searle) for 30min at 50°C. After neutralization with 50 µl of acetic acid, 10ml of a toluene-based scintillation fluid (Econofluor; NEN) was added, and the samples were counted for radioactivity in a Beckman LS-230 scintillation counter.

U.v. photoaffinity labelling

The u.v.-irradiation-induced attachment of ATP to isolated nuclear particles was performed by the procedure described by Berrios *et al.* (1983). Samples with 3 µCi of [α-³²P]ATP (1–2 µCi/pmol; 0.05–0.1 µM) in 33 µl of 10mM-Tris/HCl, pH 7.4, containing 0.1mM-MgCl₂ and 25 µg of PMSF/ml were irradiated at 0°C at a distance of 3.5cm from the plane of the u.v. source (two 25W General Electric G25T8 germicidal lamps) for 30min, then

treated with 2 μ g of RNAase A. After 10 min at 0°C the sample was mixed with 70 μ l of Sample Buffer (0.062 M-Tris/HCl, pH 6.8, 2% sodium dodecyl sulphate, 10% glycerol, 5% 2-mercaptoethanol and 0.002% Bromophenol Blue) with 1 mM-ATP, heated to 90°C for 3 min, chilled, and mixed with 40 μ l of cold 50% trichloroacetic acid. The resulting precipitate was collected by centrifugation (15000g for 5 min) and washed twice with 5% trichloroacetic acid and twice with acetone. After drying for 30 min under vacuum at room temperature, 5 μ l of 1 M-Tris base was added and the precipitate was dissolved in 50 μ l of Sample Buffer. The solution was heated to 90°C for 5 min, cooled and analysed by polyacrylamide-gel electrophoresis as described by Blanchard & Richardson (1983). Gels stained with Coomassie Blue were dried on to Whatman 3M paper, and radiolabelled proteins were localized by autoradiography with Kodak XAR-5 film exposed to the dried gel at -70°C with an intensifier screen.

Results

Ribonuclease-sensitivity of nuclear ATPase

The amount of RNA-dependent ATPase activity in nuclei can be estimated indirectly by determining the fraction of the total ATPase activity that is lost when purified nuclei are treated briefly with pancreatic ribonuclease. By this test, as much as one-third of the ATPase activity in HeLa nuclei was sensitive to RNAase. In our standard assay a typical preparation of HeLa nuclei contained 30 ($\pm$ 5) units of ATPase activity/mg of protein. When a suspension of nuclei (containing 6 μ g of DNA in 60 μ l) was treated for 5 min at 0°C with 33 μ g of RNAase/ml, the specific activity was decreased to 20 units/mg. Almost identical results were obtained when nuclei were treated with either half or twice as much RNAase or were incubated for twice as long. Thus the conditions appear to be sufficient to complete the effect of the RNAase digestion. The activity did not decrease when the nuclei were incubated at 0°C without added RNAase.

Co-extraction of ATPase with RNA

To obtain further evidence that a significant fraction of the nuclear ATPase activity depends on an association with RNA, we tested whether activity could be selectively extracted from nuclei under mild conditions that allow solubilization of RNP particles (Samarina & Krichevskaya, 1981). When purified HeLa nuclei containing pulse-labelled RNA (from cells treated with low concentrations of actinomycin to suppress incorporation of label into rRNA) were incubated at 20°C in a solution containing 0.1 M-NaCl, 1 mM-MgCl₂ and

10 mM-Tris/HCl, pH 8.5, 30% of the labelled RNA and 50% of the ATPase activity were released after 3 h (Fig. 1). The release of the RNA was a strongly temperature-dependent process; very little was released when the nuclei were incubated at 0°C. Although some (13%) of the ATPase was released immediately at both 20°C and 0°C, much of it (35%) was released with the same temperature- and time-dependence as the RNA. Thus a significant fraction of the nuclear ATPase was co-extracted from HeLa nuclei with precursors to mRNA.

This fraction could be increased substantially by treatment of the nuclei with 50 mM-Na₃EDTA (pH 8) for 5 min at 0°C before the extraction in the NaCl/MgCl₂/Tris buffer. With this procedure as much as 70–75% of the labelled RNA and of the ATPase activity, but less than 1% of the DNA,

Fig. 1. Kinetics of release of ³H-labelled heterogeneous nuclear RNA and ATPase from HeLa nuclei

HeLa cells (7.5×10^7 in 25 ml) were pretreated with 0.04 μ g of actinomycin D/ml for 20 min and then labelled for 20 min with 10 μ Ci of [³H]uridine/ml. Nuclei, isolated by the standard procedure, were suspended in 0.1 M-NaCl/1 mM-MgCl₂/10 mM-Tris/HCl, pH 8.5, at a density of 1×10^7 /ml. This suspension was incubated with gentle mixing on a rotary mixer at 20°C (○, △) or 4°C (●, ▲). At the indicated times, 150 μ l samples of nuclear suspension (which contained a total of 0.78 unit of ATPase and 82000 c.p.m. of [³H]RNA) were removed. After centrifugation for 5 min at 700g, the supernatant solutions were assayed for acid-insoluble radioactivity (○, ●) and for ATPases, (△, ▲).

were released after 2 h at 37°C. Since 70% of the released ATPase was sensitive to RNAase treatment, this extraction procedure was selective for the RNAase-sensitive ATPases. The addition of 25 µg of PMSF/ml to the extraction buffer did not affect these yields.

Sedimentation properties of ATPases extracted with RNP particles

To test whether the extracted ATPases were associated with a particular class of RNP particles, we analysed the sedimentation distribution of the isolated activity. After zone centrifugation of the HeLa nuclear extract on a sucrose gradient, the A_{254} profile showed distinct peaks at positions corresponding to sedimentation coefficients of 5–8S, 20S and 40S (Fig. 2). The peak at 40S contains core RNP particles (Beyer *et al.*, 1977); analysis of 40S material by polyacrylamide-gel electrophoresis showed that it contained the six core RNP polypeptides with M_r values from 32000 to 44000 as the predominant components (results not shown). When the sucrose-gradient fractions were assayed for ATP hydrolysis under the standard conditions [with poly(A) added], activity was broadly distributed from the region with 10S particles down to that with 50S particles (Fig. 2). The main peak in the distribution profile was at 24S and a distinct second peak was at 40S. After treatment of each fraction with RNAase, the activity, measured without poly(A), decreased in most fractions to less than 20% of that of the untreated samples (Fig. 2). The major exception was in the 8–10S region, where there was a small but distinct peak of RNAase-resistant ATPase. Most of the activity that was lost by RNAase treatment could be recovered if poly(A) was present in the assay mixture (Fig. 2), indicating that it was RNA-dependent. Except in fractions 2 and 3, very little or no activity was recovered when RNAase-treated samples were assayed with single-stranded DNA present (Fig. 2). The fact that single-stranded DNA caused significant stimulation of the activities in fractions 2 and 3 indicated that the major DNA-dependent ATPase activities in the nuclear extract were physically distinct from the RNA-dependent activities.

To test whether the physical heterogeneity of the RNAase-sensitive ATPase activity could arise in part from the action of more than one distinct enzyme with different NTP substrate specificities, we compared the sedimentation distribution of UTPase activity with that of the ATPase activity. The results (Fig. 2a) show that the ratio of the rate of hydrolysis with UTP to that with ATP varied only slightly in the fractions that contained most of the RNAase-sensitive ATP hydrolysis activity (5–12). Thus the extracts did not contain physically

Fig. 2. Sedimentation analysis of ATP- and UTP-hydrolysis activities in HeLa nuclear extract

Samples of extracts containing nuclear RNP particles were fractionated by zone centrifugation on sucrose gradients as described in the Experimental section. A sample (150 µl) of each fraction was assayed for ATP hydrolysis in the standard reaction mixture with [γ - 32 P]ATP; UTP-hydrolysis activity was measured in mixtures that contained [γ - 32 P]UTP instead of [γ - 32 P]ATP. In both cases, the amount of [32 P] P_i released after 20 min was determined by the charcoal procedure. Other samples of each fraction were treated with 33 µg of RNAase A/ml and assayed for ATP-hydrolysis activity in standard reaction mixtures with the indicated modifications. In (b): ---, A_{254} ; ATP hydrolysis with poly(A) present (●), RNAase treatment and with poly(A) absent (○), after RNAase treatment and with poly(A) present (□), and after RNAase treatment and with denatured calf thymus DNA present (18 µg/ml) instead of poly(A) (△). The data points in (a) are ratios of UTPase activity to ATPase activity.

distinct ATPases with very different substrate specificities.

Attempts were made to obtain a more homogeneous enzyme by treatments with high salt (0.4M-NaCl) or RNAase before fractionation. However, in both of these cases the treatments caused the formation of aggregates, and only small amounts of activity were recovered after centrifugation in sucrose gradients (results not shown). We have not found a way of isolating the ATPase free of

endogenous RNA activator without losing most of its activity.

Characteristics of ATPase associated with the RNP fraction

To study in further detail some of the properties of the ATPase activity associated with the 14S–50S particles, fractions containing the separated particles were pooled and concentrated by centrifugation. In a typical preparation, 15% of the RNAase-sensitive ATPase from nuclei was recovered after extraction and the two centrifugations. The partially purified material had a specific activity of 70 units/mg, which was 7-fold higher than that of RNAase-sensitive ATPase in nuclei.

The results of our characterization of activators and inhibitors of the activity with the partially purified particles are summarized in Table 1. As expected from the results with the individual gradient fractions, ATPase activity was stimulated slightly with added RNA [poly(A)] and inhibited extensively by prior treatment with ribonuclease. ATP hydrolysis was also strongly inhibited by heparin and 0.2M-NaCl, which is a characteristic of purified RNA-dependent ATPases (Bektesh & Richardson, 1981; Blanchard & Richardson, 1983). The ATP-hydrolysis activity was also partially inhibited by Na_3VO_4 and *N*-ethylmaleimide, but insensitive to PMSF. The lack of inhibition with ouabain, quercetin and efrapentin

indicates that the particles were not strongly contaminated with the plasma-membrane Na^+/K^+ -dependent ATPase or the mitochondrial F_1 ATPase (Kuriki & Racker, 1976; Cross & Kohlbrenner, 1978).

A partial analysis of the requirement for a bivalent cation indicates that with 1 mM-ATP the optimum MgCl_2 concentration was 1 mM; with 10 mM- MgCl_2 , ATP was hydrolysed at 37% of the rate with 1 mM- MgCl_2 . The activity with 1 mM- CaCl_2 was 50% of that with 1 mM- MgCl_2 , and there was no activity with 10 mM-EDTA. With 1 mM- MgCl_2 , the apparent K_m for ATP, as estimated by a standard graphical procedure (not shown), was 0.2 mM. The pH-dependence exhibited a sharp optimum at pH 5.4 and nearly constant activity at about 70% of the optimum value from pH 6 to 9. There was no activity at pH 4.4.

The isolated particles catalyse the hydrolysis of dATP and CTP with about the same rate as ATP, whereas UTP was hydrolysed somewhat less rapidly and GTP was hydrolysed 40% as rapidly as ATP (Table 2). The results also show that the hydrolysis of all five nucleotides was sensitive to

Table 1. *Inhibitors of ATPase activity of the isolated subfraction of 14S–50S particles*

The standard reaction mixtures minus poly(A) contained 2.1 μg (protein) of the isolated subfraction of 14S–50S particles in a volume of 20 μl . Incubations were for 20 min at 37°C and ADP formation was measured by conversion from $[\alpha\text{-}^{32}\text{P}]\text{ATP}$.

Addition	ADP produced (nmol)
None	2.1 $\pm$ 0.2
Poly(A) (15 $\mu\text{g}/\text{ml}$)	2.6 $\pm$ 0.3
RNAase A (33 $\mu\text{g}/\text{ml}$) (5 min at 0°C before assay)	0.5 $\pm$ 0.1
Heparin (20 $\mu\text{g}/\text{ml}$)	0.4 $\pm$ 0.1
NaCl (0.2M)	0.2 $\pm$ 0.05
Na_3VO_4 (50 μM)	1.0 $\pm$ 0.1
<i>N</i> -Ethylmaleimide (2.5 mM)	0.8 $\pm$ 0.1
Phenylmethanesulphonyl fluoride (10 $\mu\text{g}/\text{ml}$)	2.0 $\pm$ 0.2
NaCl (0.06M) plus KCl (0.02M)	1.6 $\pm$ 0.2
Ouabain (0.5 mM) with 0.6M-NaCl and 0.02M-KCl	1.6 $\pm$ 0.2
Ethanol (1%)	1.7 $\pm$ 0.2
Quercetin (15 $\mu\text{g}/\text{ml}$) with 1% ethanol	1.6 $\pm$ 0.2
Efrapentin (0.05 $\mu\text{g}/\text{ml}$)	2.1 $\pm$ 0.2

Table 2. *Nucleoside triphosphatase activities associated with isolated subfraction of 14S–50S particles*

Samples with 13 μg (protein) of the isolated subfraction of 14S–50S particles were assayed in reaction mixtures containing 0.05M-Tris/HCl, pH 7.4, 1 mM- MgCl_2 and 1 mM of the indicated nucleoside triphosphate. Untreated samples were assayed with 15 μg of poly(A)/ml, whereas RNAase-treated samples were assayed without poly(A). Incubations were for 20 min at 37°C. P_i release was measured colorimetrically.

Nucleoside triphosphate	P_i released (nmol)	
	RNAase-treated	Untreated
ATP	2.5 $\pm$ 0.5	16.9 $\pm$ 2.0
GTP	0.6 $\pm$ 0.12	6.9 $\pm$ 0.8
CTP	1.9 $\pm$ 0.4	15.1 $\pm$ 1.8
UTP*	2 $\pm$ 1	12 $\pm$ 3
dATP	3.3 $\pm$ 0.5	18.3 $\pm$ 2.0

* For a reason that we do not understand, the UTP used for these assays had an abnormally high rate of spontaneous breakdown; 3 nmol of P_i was released in the absence of enzyme with UTP, instead of the value < 1 nmol released with the other nucleotides, including the $[\gamma\text{-}^{32}\text{P}]\text{UTP}$ used for the assays reported in Fig. 2. Since all the data were corrected for the background P_i release, this greater correction with the UTP is the source of the significantly higher uncertainty in its values. This uncertainty may also account for the apparent discrepancy between the ratio of UTP hydrolysis to ATP hydrolysis of 0.7 in these data and the ratios of nearly 1.0 reported in Fig. 2.

RNAase. The fact that the extents of those sensitivities were so similar suggests that an enzyme (or enzymes) with a broad substrate specificity is responsible for the activities.

Labelling of proteins in nuclear extract with [α - 32 P]ATP

One method that can be used to identify polypeptides that might have an active site for ATP hydrolysis is direct u.v. photoaffinity labelling (Berrios *et al.* 1983). In our initial studies we found that several distinct polypeptides in the nuclear extracts were labelled by u.v.-induced cross-linking with [α - 32 P]ATP. The covalent attachment of the label to the polypeptides was dependent on irradiation, and was completely abolished by the presence of 10 mM-EDTA or a 20-fold molar excess of unlabelled ATP. To identify which of the several polypeptides in the extract might be associated with the RNAase-sensitive ATPase, cross-linking was performed on material fractionated by zone centrifugation on a sucrose gradient and concentrated by pelleting. The results

from the autoradiograph of the polyacrylamide gel used to separate the polypeptides are presented in Fig. 3. In fractions 6–13, which contained the 20S–50S particles and were thus the fractions that were most enriched for the RNAase-sensitive ATPase, 39 kDa, 47 kDa and 55 kDa polypeptides were prominently labelled. Label was associated with the 39 kDa polypeptide in fractions 6–13 and thus had the same broad distribution as the RNAase-sensitive ATPase. In contrast, the 47 kDa polypeptide was most heavily labelled in fractions 3–5 (with 10S–15S particles) and was only slightly labelled in fractions 9–13. Thus its distribution did not correlate well with that of the RNAase-sensitive ATPase. The 55 kDa peptide was very strongly labelled in fractions 7 and 8, the fractions that contained the peaks in the ATPase distribution profile. The autoradiogram shows too that label was associated with the dye front and with material at the top of the gel in all fractions. The appearance of label in those positions was dependent on u.v. irradiation and was suppressed by EDTA and by unlabelled ATP (results not shown).

Discussion

We have shown that the activity of a major component of the ATPases in nuclei from HeLa cells depends on a functional interaction with RNA. This activity is characterized by its sensitivity to RNAase treatment and its co-extraction with RNA. In extracts of autodigested nuclei it is associated with various particles with sedimentation coefficients of 10–50S. It is especially abundant with 24S and 40S particles and constitutes at least 80% of the total ATPase activity associated with particles larger than the 14S particles.

In spite of their heterogeneity in size, the 18S–50S particles were quite uniform with respect to three enzymic characteristics: their ratio of UTPase activity to ATPase activity, the extent of their re-activation with poly(A), and the absence of activation with DNA. Since different polynucleotide-dependent ATPases are known to vary considerably with respect to substrate and polynucleotide-cofactor specificities, the uniformity of these characteristics suggest that a single enzyme is responsible for most of the activity with the 18S–50S particles. The size heterogeneity could merely be the consequence of an association of the enzyme with RNA in different particles. The broad distribution of activity was found whether the serine-proteinase inhibitor PMSF was used or not. However, we have not ruled out the possibility that the action of PMSF-insensitive proteinases could be responsible for some of the heterogeneity.

Further evidence for an association of one or at most two ATPases with the various particles

Fig. 3. *U.v. photoaffinity labelling of proteins in nuclear extracts separated by zone centrifugation*

Extracts of HeLa nuclei were fractionated on three 14.3 ml sucrose gradients as described in the Experimental section. Corresponding fractions for each of the gradients were combined, and the particles were collected by centrifugation for 12 h at 45000 rev./min in the Beckman Ti 50 rotor. Each pellet was resuspended in 60 μ l of a solution containing 10 mM-Tris/HCl buffer, pH 7.4, 0.1 mM-MgCl₂ and 25 μ g of PMSF/ml. The photoaffinity treatment and gel-electrophoretic analysis were performed as described in the Experimental section. Autoradiography was for 48 h. The numbers across the top identify the fractions from the sucrose gradients. The numbers on the left are the sizes of marker polypeptides in kDa. The numbers on the right identify the 39 and 55 kDa proteins.

comes from the results of direct u.v. photoaffinity labelling of polypeptides with [α - 32 P]ATP. This method revealed a 39kDa polypeptide with the same broad distribution as the RNAse-sensitive ATPase activity and a 55kDa protein with the 24S particles. Since most ATP-binding subunits in ATPases are larger than 20kDa and smaller than 200kDa (Kornberg, 1980), we believe it is unlikely that the label associated with the dye front and material at the top of the gel is obscuring a correlation with unresolved proteins in those regions of the gel. We cannot rule out the possibility that the 39kDa and 55kDa polypeptides are components of other enzymes that bind ATP. However, we do have evidence, from a lack of coincidence in their distributions, that they are probably not components of protein kinase or a poly(A) polymerase, two other enzymes that are associated with RNP particles under certain conditions (Blanchard *et al.*, 1977; Louis *et al.*, 1978); the protein kinase activity in the gradient fractions was distributed with a distinct sharp peak at 15S, whereas poly(A) polymerase was found in the <5S region (results not shown).

Two RNA-dependent ATPases have been purified to near homogeneity from rat liver nuclei (Blanchard & Richardson, 1983). One of these enzymes, ATPase I, consists of a microheterogeneous monomeric protein of M_r 53000–60000. The other enzyme, ATPase II, has M_r 37000. The similarity in the sizes of these proteins to the sizes of the HeLa polypeptides that photo-cross-link ATP in nuclear extracts suggests that HeLa nuclei contain enzymes that are equivalent to ATPases I and II as major components. However, there are two characteristics of the purified rat liver enzymes that do not match very well with the characteristics of the activities with the HeLa nuclear particles. First, both rat liver enzymes were nearly as active with single-stranded DNA as with poly(A), whereas the HeLa nuclear particles had very little ATPase activity with DNA as a replacement cofactor. Second, the most active of the two isolated rat liver ATPases was active only with ATP and dATP, whereas the enzymes associated with the HeLa particles hydrolysed GTP, CTP and UTP almost as well as ATP and dATP. We have found that the characteristics of the NTPase activities with isolated rat liver nuclear particles are very similar to the activities with the HeLa particles (L. V. Richardson & J. P. Richardson, unpublished work). Thus the differences in properties appear to be a result of purification. Since yields were very low (<2%), the purified enzymes could be only minor components of the nuclear NTPases, or could be devoid of cofactors or regulatory subunits. In either case, further work on the purification of these NTPases

will be needed to improve yields and to obtain pure enzymes with characteristics that can be related to the activity in the partially fractionated extracts.

The fact that a major component of the nuclear NTPase activity appears to be functionally associated with RNA in a large variety of nuclear particles suggests that one or more steps in the metabolism of RNA requires chemical energy that is derived from the hydrolysis of NTPs. Two steps that are known to be dependent on ATP hydrolysis are the splicing reactions in the maturation of mRNA (Hardy *et al.*, 1984) and the transport of RNA from the nuclei to the cytoplasm (Agutter *et al.*, 1976). The initial characterizations of the nuclear ATPases reported in the present paper should assist in the identification of specific enzymes involved with important steps of nuclear RNA metabolism.

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