ACKNOWLEDGMENTS

Thanks are due to C. Van Herrewège and F. F. Clerc for the drawings.

Registry No. ATP, 56-65-5; ADP, 58-64-0; ATPase, 9000-83-3; ATP synthase, 37205-63-3.

REFERENCES

Archinard, P., Penin, F., Godinot, C., & Gautheron, D. C. (1986) *Methods Enzymol. 126*, 455-460.

Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241-250.

Di Pietro, A., Godinot, C., Bouillant, M. L., & Gautheron, D. C. (1975) *Biochimie 57*, 959-967.

Di Pietro, A., Penin, F., Godinot, C., & Gautheron, D. C. (1980) *Biochemistry 19*, 5671-5678.

Di Pietro, A., Fellous, G., Godinot, C., & Gautheron, D. C. (1986) *Biochim. Biophys, Acta 851*, 283-294.

Di Pietro, A., Penin, F., Julliard, J. H., Godinot, C., & Gautheron, D. C. (1988) *Biochem. Biophys. Res. Commun. 152*, 1319-1325.

Fellous, G., Godinot, C., Baubichon, H., Di Pietro, A., & Gautheron, D. C. (1984) *Biochemistry 23*, 5294-5299.

Frangione, B., Rosenwasser, E., Penefsky, H. S., & Pullman, M. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7403-7407.

Gautheron, D. C., Durand, R., Pialoux, N., & Gaudemer, Y. (1964) *Bull. Soc. Chim. Biol. 46*, 645-660.

Gautheron, D. C., Penin, F., Delêage, G., & Godinot, C. (1986) *Methods Enzymol. 126*, 417-427.

Godinot, C., Vial, C., Font, B., & Gautheron, D. C. (1969) *Eur. J. Biochem. 8*, 385-394.

Godinot, C., Penin, F., & Gautheron, D. C. (1979) *Arch. Biochem. Biophys. 192*, 225-234.

Gomez-Fernandez, J. C., & Harris, D. A. (1978) *Biochem. J. 176*, 967-975.

Gomez-Puyou, A., Tuena de Gomez-Puyou, M., & Ernster, L. (1979) *Biochim. Biophys. Acta 547*, 252-257.

Harris, D. A., Radda, G. K., & Slater, E. C. (1977) *Biochim. Biophys. Acta 459*, 560-572.

Harris, D. A., Von Tscharner, V., & Radda, G. K. (1979) *Biochim. Biophys. Acta 548*, 72-84.

Husain, I., Jackson, P. J., & Harris, D. A. (1985) *Biochim. Biophys. Acta 806*, 64-74.

Klein, G., Lunardi, J., & Vignais, P. V. (1981) *Biochim. Biophys. Acta 636*, 185-192.

Lippe, G., Sorgato, C., & Harris, D. A. (1988a) *Biochim. Biophys. Acta 933*, 1-11.

Lippe, G., Sorgato, C., & Harris, D. A. (1988b) *Biochim. Biophys. Acta 933*, 12-21.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891-2899.

Penefsky, H. S. (1985) *J. Biol. Chem. 260*, 13735-13741.

Penin, F., Godinot, C., & Gautheron, D. C. (1979) *Biochim. Biophys. Acta 548*, 63-71.

Penin, F., Delêage, G., Godinot, C., & Gautheron, D. C. (1986) *Biochim. Biophys. Acta 852*, 55-67.

Pullman, M. E., & Monroy, G. C. (1963) *J. Biol. Chem. 238*, 3762-3769.

Pullman, M. E., Penefsky, H. S., Datta, A., & Racker, E. (1960) *J. Biol. Chem. 235*, 3322-3329.

Schwerzmann, K., & Pedersen, P. L (1981) *Biochemistry 20*, 6305-6311.

Schwerzmann, K., & Pedersen, P. L (1986) *Arch. Biochem. Biophys. 250*, 1-18.

Nuclear Envelope Glycoprotein with Poly(A) Polymerase Activity of Rat Liver: Isolation, Characterization, and Immunohistochemical Localization†

Rabinder N. Kurl, Stephanie C. Holmes, Ethel Verney, and Herschel Sidransky*

Department of Pathology, The George Washington University Medical Center, Washington, D.C. 20037

Received March 25, 1988; Revised Manuscript Received July 28, 1988

ABSTRACT: A protein with poly(A) polymerase activity has been identified and isolated from hepatic nuclear envelopes of rats to near homogeneity. The ability of the enzyme to bind to concanavalin A–agarose and to be eluted from the column with methyl α-D-mannopyranoside (0.2 M) as well as the inhibitory effects of α-mannosidase suggested that it was a glycoprotein. Poly(A) polymerase has an absolute requirement for a divalent cation, ATP, and an oligonucleotide primer. The enzyme activity with Mn^{2+} was about 20-fold higher than that with Mg^{2+}. Several known inhibitors adversely affected poly(A) polymerase activity. The enzyme has a molecular weight of 64 000 when analyzed by polyacrylamide gel electrophoresis under denaturing conditions and has a sedimentation coefficient of 4.5 S. Immunohistochemical studies using polyclonal antibodies raised against the purified enzyme revealed that the antigen was localized in the nuclear membranes.

Polyadenylation is one of the nuclear processes involved in the maturation of hnRNA prior to its transport to the cytoplasm as mRNA. Poly(A) sequences have been implicated in (i) mRNA stability (Nudel et al., 1976), (ii) nucleocytoplasmic transport of mRNA (Muller et al., 1984), and (iii) protein synthesis (Jacobson & Favreau, 1983; Rubin & Halim, 1987). However, most histone mRNAs lack poly(A) sequences (Adesnik & Darnell, 1972; Greenberg & Perry, 1972), and also a population of mRNA isolated from adult rat brains has recently been shown to lack the sequences (Chaudhari & Hahn, 1983).

† This work was supported by U.S. Public Health Service Research Grants CA-41832 from the National Cancer Institute and DK-27339 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

0006-2960/88/0427-8974$01.50/0

Transcribed regions of actively expressed genes have been shown to be associated with the nuclear matrix (Ciejek et al., 1983). Moreover, even hnRNA and mRNA have been shown to be bound to the nuclear matrix (Miller et al., 1978; Herman et al., 1978), and this association of hnRNA is present during both transcription (Jackson et al., 1981) and the course of its processing to mRNA (Long et al., 1979).

Addition of poly(A) sequences to the 3′-terminal segment of hnRNA is catalyzed by poly(A) polymerase. This reaction was first described about 3 decades ago (Edmonds & Abrams, 1960), and since then the enzyme has been purified and characterized from several procaryotes and eucaryotes [reviewed by Edmonds (1982) and Jacob and Rose (1983)]. Though poly(A)polymerase has been reported to be of nuclear origin (both nucleoplasmic and chromosomal), no information has been available about the presence of the enzyme in nuclear envelopes. Such localization may be of prime importance in view of recent reports that both actively transcribed genes and hnRNA (undergoing processing) are located in the nuclear matrix as described above.

In this paper, we describe the presence of poly(A) polymerase in nuclear envelopes of rat liver and the purification of the enzyme therefrom. The enzyme is a glycoprotein. Localization of the enzyme in the nuclear membranes of rat liver has been established immunohistochemically using polyclonal antibodies raised against the enzyme.

Materials and Methods

Animals. Sprague-Dawley rats (Harlan Sprague Dawley Inc., Walkerville, MD) weighing 125–250 g were fed a commercial diet (Rodent Lab Chow, Purina) ad libitum and then were fasted overnight (about 18 h) prior to sacrifice.

Isolation and Subfractionation of Nuclei. Nuclei were prepared from rat liver as described by Blobel and Potter (1966). Briefly, the liver tissue was minced and homogenized in 2 volumes of buffer A (0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M $MgCl_2$, and 0.25 M sucrose). The homogenate was filtered through cheesecloth (four layers) prior to mixing with 2 volumes of buffer B (0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M $MgCl_2$, and 2.3 M sucrose). The latter was underlaid with 1 volume of buffer B and centrifuged for 60 min at 105000*g* in a Beckman L5-75 ultracentrifuge. The supernatant was discarded, and the pellet was washed once with buffer A and at least twice with buffer C (0.05 M Tris-HCl, pH 8.5, 0.05 M KCl, 0.001 M $MgCl_2$, 0.002 M EDTA, and 0.002 M DTT). The nuclei were then suspended in buffer C and fractionated according to the method described by Tata and Baker (1974).

Nuclei were sonicated (while kept cold in an ice bath) by 3 × 15 s bursts, and microscopic examination revealed that the sonication process disrupted over 90% of the nuclei. The nucleoplasm was separated by centrifugation (7000*g* for 35 min at 4 °C), and the pellet was suspended in buffer D (i.e., buffer C containing 0.09 M each of sodium and potassium citrate). The suspension was placed on a discontinuous gradient of 0.88, 1.17, 1.37, 1.72, and 2.0 M sucrose in buffer D. The gradients were centrifuged at 94000*g* average for 2 h at 4 °C. This procedure led to the recovery of euchromatin which floated on the surface of the gradient. The nuclear membranes sedimented at the 0.00/0.88 and 0.88/1.17 M interfaces, the nucleoli were present at the 1.37/1.72 M interface, and heterochromatin was present as a pellet at the bottom. Nuclear material present at interfaces 1.17/1.37 M and 1.72/2.0 M represented debris as well as less well-characterized material and hence was indicated as residual in Table I.

Preparation of Nuclear Envelopes. Nuclear envelopes were isolated according to Agutter and Gleed (1980) which is a modified technique of that described by Harris and Milne (1974) and is routinely used in this laboratory (Kurl et al., 1987).

Solubilization of Nuclear Envelopes. Nuclear envelopes were solubilized in buffer F [0.02 M Tris-HCl, pH 7.5, 0.002 M EDTA, 0.002 M dithiothreitol, 0.001 M phenylmethanesulfonyl fluoride (PMSF), and 20% v/v glycerol] containing either 1% v/v Triton X-100 for 3 h at 4 °C or 0.5% w/v CHAPS for 1 h at 4 °C. Material which remained insoluble was removed by centrifugation (200000*g* for 90 min).

Chromatography of Solubilized Nuclear Envelopes of a Concanavalin A–Agarose Column. Solubilized nuclear envelopes (i.e., 200000*g* supernatant) were diluted with buffer F [without the detergent so as to reduce the concentration of the detergent to 0.1% (v/v)]. The matrix was equilibrated with buffer F containing 0.1% (v/v) Triton X-100. The soluble proteins were incubated with the matrix for 4 h at room temperature on a rocking platform. The following day, the unbound proteins were collected, and the matrix was washed with equilibration buffer (5–10× column volumes). Bound proteins were eluted with equilibration buffer containing methyl α-D-mannopyranoside (0.2 M). Each fraction was assayed for the enzyme.

Glycerol Gradient Analysis of Enzyme Eluted from the Concanavalin A–Agarose Column. Eluate from the concanavalin A–agarose column was analyzed on linear (10–30%) glycerol gradients in buffer G [0.02 M Tris-HCl, pH 8.0, 0.002 M EDTA, 0.002 M dithiothreitol, 0.001 M phenylmethanesulfonyl fluoride (PMSF), and 0.1% (v/v) Triton X-100]. The gradients were centrifuged at 4 °C in a Beckman SW 50.1 rotor at 35000 rpm for 17 h. Protein markers of known sedimentation coefficients (bovine serum albumin, 4.5 S, and aldolase, 7.8 S) were centrifuged at the same time through identical gradients and were located by their absorbance at 280 nm.

Electrophoresis of Proteins Eluted from the Concanavalin A–Agarose Column. Eluate from the concanavalin A–agarose column was concentrated by using a Minitan system (Millipore, MA) prior to precipitation with trichloroacetic acid (10%). The proteins were washed with acetone, suspended in denaturing buffer, placed in boiling water for 5 min, and analyzed on polyacrylamide gels (10%) under denaturing conditions as described by Laemmli (1970). A silver stain (ICN) was used to identify proteins on the gel.

Preparation of Antibodies against the Enzyme. The protein with an appropriate molecular weight, visible after polyacrylamide gel electrophoresis under denaturing conditions as described earlier, was excised from the gel, homogenized in phosphate-buffered saline (PBS), combined with the adjuvant (MPL + TDM emulsion; RIBI Hamilton, MT), and injected into rabbits (New Zealand white) intramuscularly. Thereafter, booster injections were given at weekly intervals for 5 weeks. A total amount of approximately 400 μg of the antigen was injected. The rabbits were bled 2 weeks after the final injection, and the serum was assayed for antibodies against the antigen using ELISA and the Western blot techniques (Towbin et al., 1979). The rabbits were bled prior to the injection of the antigen, and the serum obtained was used for control studies.

Immunohistochemical Localization of the Enzyme. Livers removed from rats after decapitation were quickly frozen, and 5-μm sections were cut in a cryostat and thaw-mounted onto glass slides for immunohistochemistry. The tissue sections were

Table I: Poly(A) Polymerase Activity in Various Nuclear Fractions of Rat Liver[a]

fraction	volume (mL)	activity		total protein (mg)	sp act.
		total (cpm)	recovered (%)		
euchromatin	20	32888 ± 10232[c]	45	4.23	7775
nuclear envelopes	27	32949 ± 1980	45	12.3	2679
heterochromatin	2	7634 ± 2695[d]	10	6.5	1174
nucleoli	12	ND[b]		0.25	
residual	30	ND		0.58	

[a] Rat hepatic nuclei were sonicated, and the nucear sap was separated by centrifugation. The pellet was suspended in citrate buffer and layered on a discontinuous gradient containing 0.88, 1.17, 1.37, 1.72, and 2.0 M sucrose in citrate buffer. Contents of four tubes were pooled. Nuclear envelopes separate out at the 0.00/0.88 and 0.88/1.17 M interfaces; the nucleoli are at the 1.37/1.72 M interface. Euchromatin floats on the surface of the gradient whereas heterochromatin is present as a pellet at the bottom of the tube. Material at the 1.17/1.37 and 1.72/2.0 M interfaces which has not been characterized accounts for residual activity. [b] ND, not detectable. [c] SEM (n = 5). [d] In two experiments, no activity was detectable.

Table II: Purification of Poly(A) Polymerase from Rat Liver Nuclei and Nuclear Envelopes

fraction	volume (mL)	total protein (mg)	total act. (cpm)	yield[b] (%)	sp act.[c]	purification (x-fold)
nuclei	135	449	2 580 403	100	5 655 ± 870	
nuclear envelopes	18	162	982 692	38	6 066 ± 1 358	1.07
solubilized	20	15.6	495 581	19.2	31 768 ± 9 999	5.6
Con A eluate	6[a]	0.11	28 111	1.1	187 317 ± 26 851	31

[a] The dilute (50 mL) eluate was concentrated with Amicon B15 to obtain a final volume of 6 mL, aliquots of which were used for the enzyme assay. [b] Yield is from one typical experiment. [c] Specific activity is the mean ± SEM (n = 5).

permeabilized with acetone at −20 °C for at least 3 min. The slides were washed with PBS at room temperature 3 times for 5 min each. The sections were exposed to either the preimmune serum (1:500 in PBS) or the serum containing the antibody (1:500 in PBS) for 3 h at room temperature. The slides were washed as described previously and incubated with peroxidase-labeled second antibody (1:250 in PBS) for 1 h at room temperature. The slides were washed as before and incubated with 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/mL) containing 0.01% H_2O_2 in Tris buffer, pH 7.6. The sections were viewed under the microscope after prior rinsing and counter-staining with Malachite Green.

Enzyme Activity. Poly(A) polymerase activity was measured as described by Jacob et al. (1976).

Protein Determination. The protein content of the various fractions after precipitation with trichloroacetic acid was determined as described by Lowry et al. (1951).

Results

The presence of poly(A) polymerase in cell nuclei has been well established (Jacob & Rose 1983). Moreover, the enzyme is known to be present in two forms, free and bound. The free form is present in the nucleoplasm (nuclear sap), whereas the bound form is associated with chromatin. In order to determine whether the enzyme is present in other nuclear components, e.g., nuclear envelopes and nucleoli, rat hepatic nuclei were isolated, sonicated, and then fractionated as described by Tata and Baker (1974). As demonstrated in Table I, of the recovered total enzymatic activity, about half was present in the fractions containing euchromatin and nuclear envelopes, respectively, and the remaining 10% was found in the fraction containing heterochromatin. Nucleoli and uncharacterized nuclear material contained no enzymatic activity.

Since the fraction containing nuclear envelopes demonstrated appreciable enzyme activity, further experiments were performed to purify the enzyme from solubilized nuclear envelopes. Preliminary experiments (data not presented) revealed that α-mannosidase could decrease the enzymatic activity of nuclear envelopes, suggesting that mannose residues were present in the enzyme molecule. Therefore, an attempt was made to purify the enzyme by using an affinity matrix, concanavalin A–agarose (con–A), which has an affinity for mannose residues. Solubilized nuclear envelopes were layered

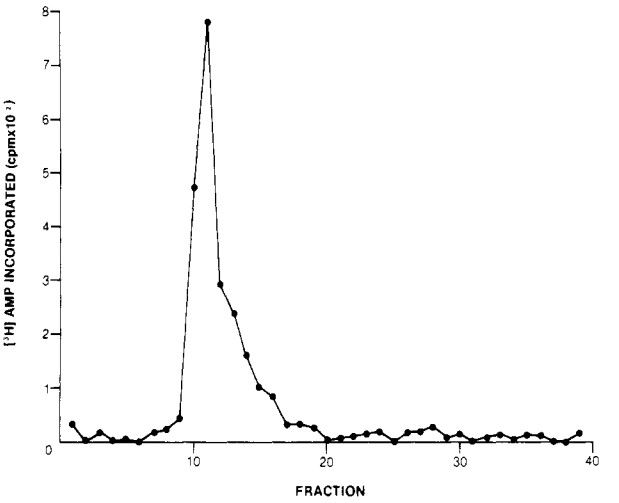

FIGURE 1: Elution profile of poly(A) polymerase on a concanavalin A–agarose column. Solubilized nuclear envelopes were chromatographed on a concanavalin A–agarose column, the column was washed with at least 5 × bed volumes of equilibration buffer, and bound proteins were eluted with buffer containing methyl α-D-mannopyranoside (0.2 M). Aliquots (80 μL) of each fraction were assayed for enzymatic activity.

on a con–A column, and the bound proteins were eluted with methyl α-D-mannopyranoside (mannose–0.2 M). Each of 39 column fractions was assayed for enzymatic activity, a profile of which is shown in Figure 1. During the course of the purification, the enzymatic activities of the nuclei and of nuclear envelopes (before or after solubilization as well as its con–A eluate) were monitored (Table II). Relative to its presence in hepatic nuclei, the enzyme was purified about 33-fold. In subsequent experiments, the starting material was usually nuclear envelopes of rat liver, which in itself contributed to the purity of the enzyme. The low yield of the enzyme can be partially explained due to adsorption of the protein to the concentrators (in this case, Amicon B15) which are known to bind proteins. Inclusion of Zn^{2+} (either Cl^- or CH_3COO^-) in the enzyme assay increased the enzymatic activity of the eluate severalfold (data not shown), suggesting the role of the metal as a catalyst.

Analysis of the proteins bound to con–A on polyacrylamide gels under denaturing conditions revealed after silver staining

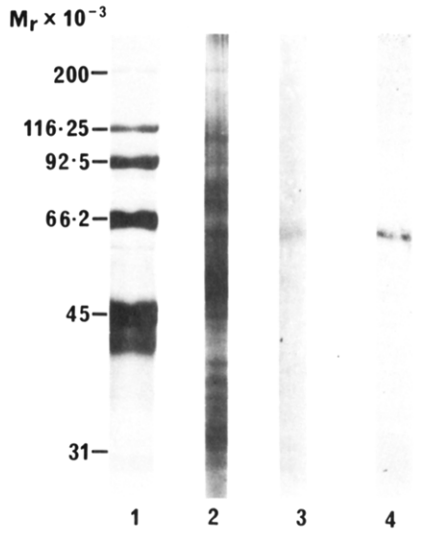

FIGURE 2: Electrophoresis of proteins eluted from a concanavalin A–agarose column under denaturing conditions. Proteins were precipitated with trichloroacetic acid, washed with acetone, denatured, and separated on a polyacrylamide (10%) slab gel using conditions described by Laemmli (1970). Molecular weight markers (lane 1), solubilized nuclear envelopes (lane 2), the concanavalin A–agarose eluate (lane 3), and an immunoblot of lane 2 (lane 4). The proteins on the gel were identified with silver stain (ICN).

Table III: Specificity of Poly(A) Polymerase for Various Nucleoside Triphosphates, Oligonucleotides, and Polynucleotides[a]

unlabeled addition	oligo-/polynucleotide added	enzymatic act. (%)
ATP	poly(A)	100
CTP	poly(A)	204
GTP	poly(A)	89
UTP	poly(A	154
ATP	poly(G)	78
ATP	poly(U)	13
ATP	$(Ap)_3A$	32
ATP	tRNA	21

[a] Activity of the purified enzyme was measured in the presence of radioactive ATP and various unlabeled nucleoside triphosphates (0.33 mM), oligonucleotides (15 μg/mL), and polynucleotides (125 μg/mL). A parallel set of tubes containing the purified enzyme and cofactors with oligo-/polynucleotide excluded was run in each assay, and the radioactivity observed was subtracted from corresponding tubes containing oligo-/polynucleotide. 100% activity = 246 667 ± 14 848 cpm/mg of protein.

the presence of a single protein which had a molecular weight of approximately 64 000 (Figure 2). Western blot analysis, using antibodies raised against this antigen, identified a polypeptide with a similar molecular weight, suggesting the specificity of the antibodies (Figure 2).

To determine which factors were essential for the purified protein to demonstrate enzymatic activity, several parameters were investigated. The enzyme had an absolute requirement for an oligonucleotide, ATP, and a divalent cation. When equimolar concentrations were used (0.01 M), the effect of Mn^{2+} on enzymatic activity was about 20-fold higher than that with Mg^{2+} (Mn^{2+} = 228 000 cpm of [^{3}H]AMP incorporated/mg of protein; Mg^{2+} = 12 000 cpm/mg of protein). Also, the specificity of the purified enzyme for ATP and other nucleoside triphosphates as substrate was investigated. With radioactive ATP, the inclusion of unlabeled GTP slightly reduced the incorporation of AMP while the inclusion of CTP or UTP markedly increased the incorporation of AMP (Table III).

Several oligo- and polynucleotides were investigated for their effect on poly(A) polymerase activity. Of the oligonucleotides, $(Ap)_3A$ and tRNA reduced enzymatic activity (Table III).

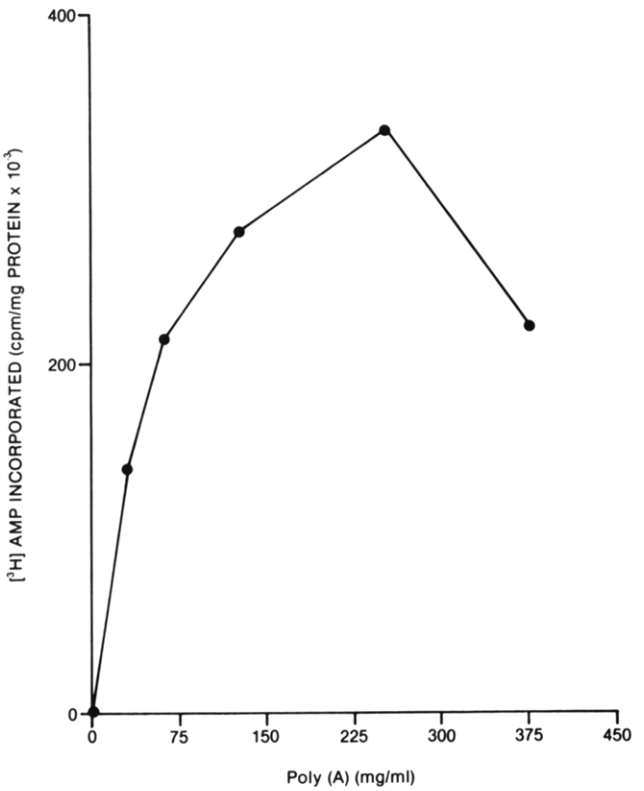

FIGURE 3: Effect of concentration of poly(A). The enzymatic activity of the purified poly(A) polymerase was investigated by using five different concentrations of poly(A). Control tubes were supplemented with an equivalent volume of water. No activity was detected in the absence of poly(A).

Table IV: Effect of Various Chemicals on Purified Poly(A) Polymerase Activity[a]

chemical	concn	enzymatic act. (%)
rifamycin AF/013	100 μg/mL	72
proflavin	100 μg/mL	51
cordycepin triphosphate	100 μg/mL	74
adenosine, 5′-(α,β-methylenetriphosphate)	1.45 mM	25
spermine	1 mM	0
1,10-phenanthroline	0.1 mM	0
sodium vanadate	1 M	0
sodium phosphate dibasic	35 mM	0

[a] Enzymatic activity (100%, control) was measured as described under Materials and Methods. Each chemical was dissolved in an appropriate solvent, an equal volume of which are added to the control tubes. A parallel set of tubes containing the purified enzyme and cofactors with poly(A) excluded was run in each assay, and the radioactivity observed was subtracted from corresponding tubes containing poly(A). Control value (100%) = 232 000 ± 4042 cpm/mg of protein.

While the enzyme activity was pronounced in the presence of poly(A) (125 μg/mL), substitution by poly(G) caused somewhat diminished enzyme activity, and substitution by poly(U) caused a marked decrease of enzyme activity (Table III). In a subsequent experiment, enzymatic activity was slightly increased (16%) with 250 μg/mL poly(A) (Figure 3).

A linear relationship was evident between the incubation time (0–90 min) and the incorporation of AMP at 37 °C (data not shown). Incubation for 60 min at 37 °C yielded appreciably higher enzymatic activity than after 60-min incubations at lower temperatures, i.e., 22 °C (room temperature) and 4 °C (data not shown).

The effects of the addition of each of several chemical compounds on poly(A) polymerase activity are illustrated in Table IV. While rifamycin, proflavin, cordycepin triphosphate

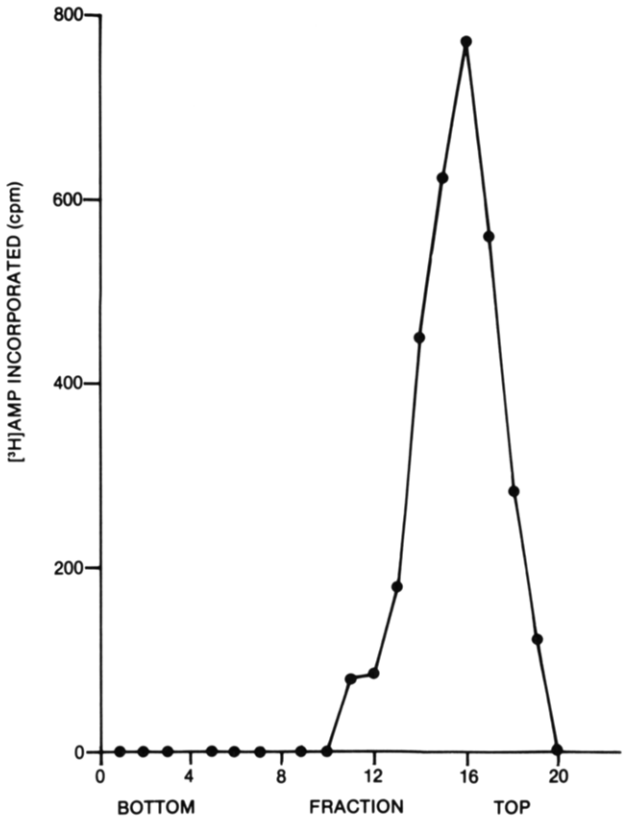

FIGURE 4: Sedimentation analysis of purified poly(A) polymerase on glycerol gradients. The purified enzyme was layered on a 4.5-mL linear glycerol gradient (10–30%) and centrifuged for 17 h at 35 000 rpm (SW 50.1 Beckman). Fractions (10 drops) were collected from the bottom of each tube and then assayed for enzymatic activity. The position of bovine serum albumin (4.5 S) was located by measuring the absorbance of each fraction at 280 nm (fraction 16).

and adenosine 5′-(α,β-methylenetriphosphate) inhibited the activity to varying degrees (26–75%), complete inhibition was observed with sodium phosphate dibasic, sodium vanadate, spermine, and 1,10-phenanthroline.

To determine the sedimentation coefficient of poly(A) polymerase, the purified enzyme was subjected to glycerol gradient analysis. As shown in Figure 4, the peak of enzymatic activity was observed in fraction 16, which coincided with the fraction which contained the peak of BSA when fractions were measured for optical density at 280 nm.

Table V: Effect of α-Mannosidase on Purified Poly(A) Polymerase Activity[a]

concn of α-mannosidase (units/mL)	enzymatic act. (%)
boiled	100, control
0.6	51
1.2	40
2.4	0

[a] Eluate from the concanavalin A-agarose column was concentrated by using a Minicon type B15 (Amicon) concentrator with a molecular weight cutoff of 15 000. Methyl α-D-mannopyranoside used for the elution of proteins from the column was removed from each sample as it would have interfered with the effect of α-mannosidase. The control value (100%) varied from 68 000 to 108 000 cpm/mg of protein. As controls, equivalent amounts of α-amnnosidase were boiled for 30 min and cooled to 4 °C by placing in ice before the addition of the purified enzyme. Other tubes were placed in ice during this period. The tubes were then incubated at 37 °C for 30 min prior to the poly(A) polymerase assay as described under Materials and Methods. Control (100%) value = 88 000 ± 11 570 cpm/mg of protein.

In order to further substantiate that the enzyme contained a carbohydrate moiety, the purified protein was treated with several concentrations of α-mannosidase. As shown in Table V, increasing amounts of α-mannosidase decreased the incorporation of AMP, suggesting that mannose residues were involved in the enzyme molecule. However, contamination of α-mannosidase by proteases cannot be ruled out in spite of the fact that PMSF was included in buffers used for the enzymatic assay.

The effect of the polyclonal antibodies on poly(A) polymerase activity was also examined. As immune IGG did not inhibit the enzymatic activity (failure to recognize enzymatic site) (data not shown), the purified poly(A) polymerase was incubated with either preimmune or immune serum (1:1000 dilution) for 2–4 h at room temperature. Thereafter, protein A–Sepharose (0.1 volume) was added, and the tubes were incubated at 4 °C for 30 min. After centrifugation, the supernatant was assayed for enzymatic activity. After 3 h of incubation, about 30% of the activity was removed by the immune serum (Table VI) while incubation for 4 h removed 87% of the activity. If, however, the incubation was performed at 4 °C for 18 h, only 45% of the enzymatic activity was removed (data not shown).

Immunohistochemical staining of liver sections showed that only the periphery of the nucleus, i.e., the nuclear rim, was stained (Figure 5). These results affirm that the major source

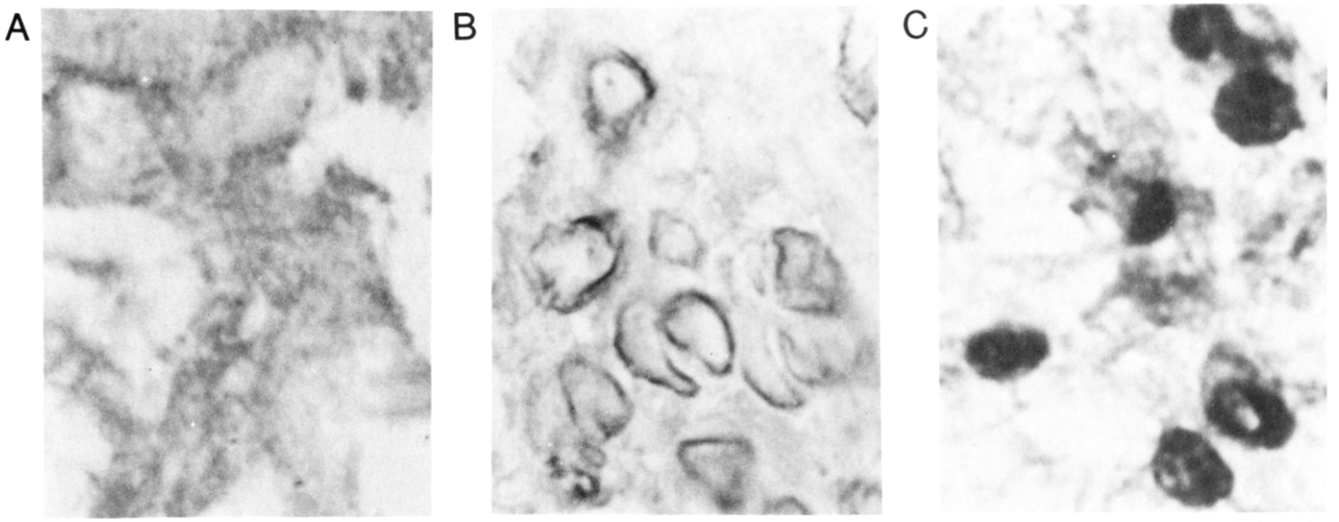

FIGURE 5: Immunohistochemical localization of the antigen. Sections of the rat liver were treated with either preimmune serum (A) or the immune serum (B) prior to immunoperoxidase staining as described under Materials and Methods. (C) Rat liver section stained with hematoxylin and eosin. Magnifications: 1960×.

Table VI: Effect of Rabbit Serum Containing Anti-Poly(A) Polymerase Antibodies on Purified Poly(A) Polymerase[a]

serum	time of incubation (h)	enzymatic act. (%)
preimmune	2–4	100
immune	2	81
immune	3	71
immune	4	13

[a]Serum (preimmune/immune) was added to a final dilution of 1:1000. After incubation at room temperature, protein A–Sepharose (0.1 volume), was added and the incubation continued at 4 °C for 30 min. Thereafter, protein A–Sepharose was sedimented by centrifugation in a microfuge for 2 min. There was no appreciable difference observed in the enzymatic activity in the presence of preimmune serum when incubated 2–4 h. Control value (100%) = 487 000 ± 27 350 cpm/mg of protein.

of the antigen was indeed the nuclear envelopes.

Discussion

A glycoprotein, with a molecular weight of approximately 64 000 and which demonstrates poly(A) polymerase activity, has been purified to apparent homogeneity. This is the first report to establish that poly(A) polymerase is a glycoprotein.

The molecular weight of poly(A) polymerase of nuclear envelopes of rat liver reported under Results is similar to that obtained by purification of nuclei of NIH–Swiss mouse embryos (Hadidi & Sethi, 1976) as well as that derived by isolation from maize (Troy & Mans, 1987) but is at variance with other published reports. Initially, Rose and Jacob (1976) assigned a molecular weight of 48 000 to the enzyme obtained from rat liver nuclei, but recently, a molecular weight corresponding to 36 000–38 000 has been reported (Stetler & Jacob, 1984). The molecular weight of the enzyme purified from other tissues and cells ranged from 37 000 to 75 000 as assessed by polyacrylamide gel electrophoresis under denaturing conditions (Edmonds, 1982). The discrepancy in molecular weights among different investigators can be attributed to a number of factors, including method of isolation, nonspecific aggregation, and source of starting material. In our laboratory, nuclear envelope proteins were observed to be more unstable than other cellular proteins, even in the presence of glycerol (20% v/v). In our early experiments, when protease inhibitors were not included in all buffers, we were able to purify a glycoprotein with a molecular weight of 33 000–34 000. However, with the inclusion of protease inhibitors, we have observed the presence of a glycoprotein with a molecular weight of 64 000. Analysis of the proteins recognized by our prepared antibodies with the Western blot technique revealed the recognition of a polypeptide with a molecular weight of 64 000. The antibodies also recognized the glycoprotein with the lower molecular weight (33 000–34 000; data not shown) prepared without protease inhibitors. Thus, the protein with the lower molecular weight isolated by others and by us when not using protease inhibitors is probably a proteolytic fragment rather than a monomer of the higher molecular weight protein. Furthermore, after prolonged storage of the enzyme, even at −70 °C, peptides of smaller molecular weight, 30 000–34 000, were observed. The phenomenon of decrease in molecular weight during storage has also been observed by others (Stetler et al., 1984). It is probable that during the isolation of nuclear envelopes a proteolytic enzyme is either released or activated which then degrades the glycoprotein.

Multiple forms of the enzyme poly(A) polymerase have been reported (Niessing, 1975). These different forms may be due to the varying amounts of the carbohydrate content of the enzyme. Glycosylation and deglycosylation may be a mechanism of controlling enzymatic function.

The enzyme purified from the nuclear envelopes was further characterized by using several inhibitors which are known to affect poly(A) polymerase activity. As shown in Table IV, several compounds and substrate analogues inhibited the enzymatic activity. Inorganic phosphate has also been reported to be an inhibitor of the enzyme of calf thymus nuclei (Winter & Edmonds, 1973), thus corroborating our findings that sodium phosphate dibasic inhibited enzymatic activity. As observed with other poly(A) polymerases, cordycepin triphosphate (Edmonds, 1982; Jacob & Rose, 1983) and adenosine 5′-(α,β-methylenetriphosphate) (Moore & Sharp, 1985) had inhibitory effects in our study. Antibiotics like the *o*-*n*-octyloxime of 3-formylrifamycin SV, AF/013, intercalating drugs like proflavin, naturally occurring polyamines such as spermine, and 1,10-phenanthroline all inhibited the Mn^{2+}-activated poly(A) polymerase activity to varying degrees. In the absence of poly(A), no incorporation of radioactivity was observed, suggesting that the enzyme has an absolute requirement for polyribonucleotide primer. Thus, the enzyme solubilized from nuclear envelopes is indeed poly(A) polymerase.

Localization of the enzyme in tissue sections has been observed with the use of antibodies. It is apparent (Figure 5) that the antigen is present mainly on the nuclear membrane and thus corroborates our premise based on biochemical analysis that the enzyme can indeed be isolated from the nuclear envelopes. It is also known that rat hepatic nucleoplasm lacks mannose-containing glycoproteins (Kawasaki & Yamashina, 1972).

We are not aware of any published reports indicating that antibodies have been raised against the liver nuclear enzyme in spite of the fact that one laboratory has made several attempts (Stetler & Jacob, 1984). Our success in raising antibodies may be partly due to the adjuvant. Furthermore, immunoblotting techniques revealed that the molecular weight of the protein recognized by the antibodies was the same as that seen on polyacrylamide gels run under denaturing conditions and identified with a silver stain. These results also suggest that the antibodies are specific for the antigen.

Recently, a 64-kDa nuclear protein has been identified in extracts of HeLa cells which plays a role in site-specific polyadenylation (Wilusz & Schenk, 1988). This protein has been partially purified by using DEAE-cellulose chromatography. The protein which is present in the void volume contains poly(A) polymerase activity. However, bound protein eluted with high salt does not contain poly(A) polymerase activity. It is probable that the protein present in the void volume is similar to that purified by us. Generally, glycoproteins do not bind to DEAE-cellulose, and, if they do, low concentrations of salt can easily dislodge them. Thus, the 64-kDa protein from extracts of HeLa cells may represent isoforms of poly(A) polymerase since it is known that both glycosylation (this paper) and phosphorylation (Jacob & Rose, 1983) can alter the enzymatic activity. Also, a 63-kDa protein has been purified from maize which has both poly(A) polymerase and splicing activities (Troy & Mans, 1987). It has also been demonstrated that antiserum against the small nuclear ribonucleoprotein particle (snRNP) containing U1 RNA can inhibit in vitro the polyadenylation reaction as well as the splicing reaction (Moore & Sharp, 1985). Whether the 64-kDa protein isolated by us has splicing activity and is recognized by human antisera specific for U1 snRNPs is under investigation. Perhaps, cloning the gene for the enzyme should resolve some of the observed discrepancies as well as shed light on the role that the enzyme plays in processing of mRNA (Sharp, 1987).

Registry No. Poly(A) polymerase, 9026-30-6.

REFERENCES

Adesnik, M., & Darnell, J. E. (1972) *J. Mol. Biol. 67*, 397–406.

Agutter, P. S., & Gleed, C. D. (1980) *Biochem. J. 192*, 85–89.

Blobel, G., & Potter, V. R. (1966) *Science (Washington, D.C.) 154*, 1662–1665.

Chaudhari, N., & Hahn, W. E. (1983) *Science (Washington, D.C.) 220*, 924–928.

Ciejek, E. M., Tsai, M. J., & O'Malley, B. W. (1983) *Nature (London) 306*, 607–609.

Edmonds, M. (1982) *Enzymes (3rd Ed.) 15*, 217–244.

Edmonds, M., & Abrams, R. (1960) *J. Biol. Chem. 235*, 1142–1149.

Greenberg, J. R., & Perry, R. P. (1972) *J. Mol. Biol. 72*, 91–98.

Hadidi, A., & Sethi, V. S. (1976) *Biochim. Biophys. Acta 425*, 95–109.

Harris, J. R., & Milne, J. F. (1974) *Biochem. Soc. Trans. 2*, 1251–1253.

Herman, R., Weymouth, L., & Penman, S. (1978) *J. Cell Biol. 78*, 663–674.

Jackson, D. A., McCready, S. J., & Cook, P. R. (1981) *Nature (London) 292*, 552–555.

Jacob, S. T., & Rose, K. M. (1983) in *Enzymes of Nucleic Acid Synthesis and Modification* (Jacob, S. T., Ed.) Vol. II, pp 135–157, CRC, Boca Raton, FL.

Jacob, S. T., Roe, F. J., & Rose, K. M. (1976) *Biochem. J. 153*, 733–735.

Jacobson, A., & Favreau, M. (1983) *Nucleic Acids Res. 11*, 6353–6368.

Kawasaki, T., & Yamashina, I. (1972) *J. Biochem. (Tokyo) 72*, 1517–1525.

Kurl, R. N., Verney, E., & Sidransky, H. (1987) *Nutr. Rep. Int. 36*, 669–676.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Long, B. H., Huang, C. Y., & Pogo, A. O. (1979) *Cell (Cambridge, Mass.) 18*, 1079–1090.

Lowry, O. H., Rosebrough, M. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Miller, T. E., Huang, C. Y., & Pogo, A. O. (1968) *J. Cell Biol. 76*, 675–691.

Moore, C. L., & Sharp, P. A. (1985) *Cell (Cambridge, Mass.) 41*, 845–855.

Muller, W. E. G., Agutter, P. S., Bernd, A., Bachmann, M., & Schroder, H. C. (1984) in *The 1984 Sandoz Lectures in Gerontology* (Bergener, M., Ermini, M., & Staehelin, H. B., Eds.) pp 21–57, Academic, New York.

Niessing, J. (1975) *Eur. J. Biochem. 59*, 127–135.

Nudel, U., Soreq, H., Littauer, U. Z., Marbaix, G., Huez, G., Leclercq, M., Hubert, E., & Chantrenne, H. (1976) *Eur. J. Biochem. 64*, 115–121.

Rose, K. M., & Jacob, S. T. (1976) *Eur. J. Biochem. 67*, 11–21.

Rubin, H. N., & Halim, M. N. (1987) *Biochem. Biophys. Res. Commun. 144*, 649–656.

Sharp, P. A. (1987) *Science (Washington, D.C.) 235*, 766–771.

Stetler, D. A., & Jacob, S. T. (1984) *J. Biol. Chem. 259*, 7239–7244.

Stetler, D. A., Wrenshall, L. E., Park, Y., & Jacob, S. T. (1984) *Carcinogenesis (London) 5*, 1363–1366.

Tata, J. R., & Baker, B. (1974) *Exp. Cell Res. 83*, 111–124.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Troy, R. M., & Mans, R. J. (1987) *78th Annual Meeting of the American Society of Biological Chemists*, Philadelphia, PA, Abstr. 516.

Wilusz, J., & Schenk, T. (1988) *Cell (Cambridge, Mass.) 52*, 221–228.

Winter, M., & Edmonds, M. (1973) *J. Biol. Chem. 248*, 4756–4762.