Association of Poly(A) Polymerase with U1 RNA*

(Received for publication, April 8, 1986)

Vulapalli S. Raju and Samson T. Jacob
From the Department of Pharmacology and Cell and Molecular Biology Center, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Previous studies (Stetler, D. A., and Jacob, S. T. (1984) J. Biol. Chem. 259, 7239–7244) have shown that poly(A) polymerase from adult rat liver (liver-type) is structurally and immunologically distinct from the corresponding rat hepatoma (tumor-type) enzyme. When hepatoma 7777 (McA-RH 7777) cells were labeled with [32P]inorganic phosphate, followed by immunoprecipitation with anti-hepatoma poly(A) polymerase antibodies and analysis of the RNAs in the immunoprecipitate, only one labeled small nuclear RNA corresponding to U1 RNA was found. Preimmune sera did not form a complex with U1 RNA. Hepatoma poly(A) polymerase antiserum did not immunoprecipitate U1 RNA or any other small nuclear RNA from a cell line (H4-11-EC3) which does not contain the tumor-type poly(A) polymerase. Immunoblot analysis of hepatoma 7777 nuclear extract or purified poly(A) polymerase with anti-ribonucleoprotein antisera did not show any cross-reactivity of the latter sera with poly(A) polymerase. The major RNA immunoprecipitated from the hepatoma nuclear extracts using trimethyl cap (m3G) antisera corresponded to the RNA immunoprecipitated with poly(A) polymerase antisera. These data indicate that U1 RNA is closely associated with poly(A) polymerase and suggest the potential involvement of this RNA in the cleavage/polyadenylation of mRNA precursor.

In eukaryotes, specific endonucleolytic cleavage of pre-mRNAs and poly(A) addition at the cleavage site are required for the production of functional mRNAs (for reviews, see Refs. 1–4). Perhaps the most convincing role for poly(A) is in the control of gene expression, as poly(A) addition at different sites on a single mRNA molecule can generate alternate forms of proteins (for review, see Ref. 5).

Considerable progress has been made in elucidating the signals for the cleavage and polyadenylation reactions. Two sequences in the pre-mRNA, the hexanucleotide sequences obligatory to the endonucleolytic cleavage and poly(A) addition, the biochemical nature of these reactions is not fully understood. Since anti-Sm or anti-(U1) RNP antisera or antisera to the nuclear antigen La can inhibit site-specific polyadenylation of adenosine L3 mRNA, the possible involvement of one or more small nuclear RNAs (snRNAs) in mRNA polyadenylation has been suggested. Probing in vitro polyadenylation reactions with antibodies specific for snRNPs has shown that a factor with the properties of an Sm snRNP is associated with the AUAUUAA polyadenylation signal (8). Subsequent studies (14) have shown that pretreatment of the nuclear extract with micrococcal nuclease can inhibit cleavage and polyadenylation but could be restored by the addition of purified Escherichia coli RNA. Partial cleavage of U1, U2, or U4 RNAs did not inhibit polyadenylation (15). Since none of these treatments results in complete degradation of snRNAs, it remains a possibility that other regions of the snRNA(s) may play a role in the cleavage and/or polyadenylation (15).

Our laboratory has been involved in the characterization of poly(A) polymerases (16–18). Antibodies raised against nuclear poly(A) polymerase from a rat hepatoma have been shown to form a distinct immune complex in immunoblot analysis using relatively crude nuclear extract or a purified enzyme preparation (19). Addition of these antibodies to an in vitro polyadenylation system has resulted in inhibition of polyadenylation of adenosine L3 mRNA whereas control serum had no effect indicating a direct role of poly(A) polymerase in this post-transcriptional reaction. To determine whether poly(A) polymerase is associated with any snRNA, 32P-labeled nuclear extracts derived from H4 or 7777 hepatoma cells were immunoprecipitated with anti-hepatoma poly(A) polymerase antibodies, and the immune complex was analyzed for snRNAs. The only snRNA in the complex was U1 RNA.

MATERIALS AND METHODS

Maintenance of the Cell Lines—Rat hepatoma cell lines McA-RH 7777 (7777) and H4-11-EC3 (H4) stocks were generously provided by Dr. H. S. Isom. The cell lines were maintained at 37°C in 5% CO2 on Swimms S-77 (GIBCO) containing 4 mM glutamine, 20% horse serum, 5% fetal calf serum, penicillin (35 μg/ml), and streptomycin (37 μg/ml); cell lines are subcultured weekly in T-75 flasks.

Preparation of Nuclear Extract—Cells were seeded at a concentration rate that allowed them to attain confluency in 5–6 days. At 75% confluency, the cells were grown in RPMI 1640 phosphate-free medium for 4 h. The cells were then grown in RPMI 1640 phosphate-free media containing [32P]inorganic phosphate (10 μCi/dish) for 16 h. The cells were washed with PBS twice and collected by scraping with rubber cell policeman, and nuclear extract was prepared essen-

* This work was supported by United States Public Health Service Grants CA 25078 and CA 31894 (to S. T. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: RNP, ribonucleoprotein; sn, small nuclear; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; bp, base pairs.

2 M. Terns, A. Dixit, and S. Jacob, manuscript in preparation.
tially as described (20), except that the cells were homogenized in 2 ml or 2 volumes of solution A.

Serum—Antinuclear antibodies reference human sera against U1 RNP and snRNP were obtained from the immunology branch of the Center for Disease Control, Atlanta, GA. Rabbit antisera raised against hepatoma poly(A) polymerase were used in these studies. The rabbit antisera were purified on DEAE Affi-Gel blue as specified by the manufacturer's recommendations. The flow-through fraction was saturated with ammonium sulfate and dialyzed against 0.02 M Tris-HCl, pH 8.0 and stored at -20 °C.

Immunoblot Analysis—Aliquots of nuclear extract or hepatoma poly(A) polymerase purified essentially to homogeneity (16, 19) were precipitated with equal volumes of acetone on ice for 1 h. The pellets were washed once with 100 µl of acetone and dried under vacuum in a Speedvac for 5 min. The proteins were dissolved in SDS gel sample buffer, heated for 10 min at 90 °C and subjected to electrophoresis on 10% SDS-polyacrylamide gel as described (21), and transferred to nitrocellulose sheets (Hybond C; Amersham Corp.) by electrophoresis at room temperature at 100 mA for 13–15 h in a buffer containing 25 mM Tris-HCl, pH 8.3, 0.15 M glycerol, and 10% methanol. Antigenic proteins were probed with different antisera as described (22) with some modifications using a Vector ABC staining kit. Marker lines were cut and stained with Amido Black. Nitrocellulose sheets were washed with PBS for 5 min, and the sheets were then incubated with PBS containing 5% dry milk powder at room temperature to block the nonspecific binding. The sheets were washed with PBS for 5 min, and the sheets were then incubated with anti-poly(A) polymerase antisera (1:25 dilution), human control sera, or anti-U1 antisera (1:100) in PBS-milk for 12–15 h. The blots were washed five times with PBS and incubated with biotinylated goat antirabbit IgG (1:400 dilution) in PBS-milk for 2 h at room temperature. After five washes with PBS, the sheets were incubated with avidin horseradish peroxidase DH reagents from the Vectastain ABC kit for 30 min at room temperature. The blots were washed once for 5 min with PBS containing 0.1% Triton X-100 and four times with PBS. The blots were then incubated with 100 ml of PBS solution containing 60 mg of 4-chloro-1-naphthol and 60 µl of 30% hydrogen peroxide.

Analysis of Immunoprecipitated RNA—The radiolabeled nuclear extracts were incubated for 30 min with 0.5 volumes of 10% Pansorbin solution (Calbiochem) in NET-2 (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.05% Nonidet P-40) at 0 °C (23) and centrifuged to remove Pansorbin. The immunoprecipitation was carried out with 10–20 µl of various antibodies coated onto 3 mg of protein A-Sepharose (Sigma) as described (23). The antibody-coated protein A-Sepharose beads were incubated at room temperature with 300 µl of nuclear extract (obtained from approximately 1.43 x 10⁷ hepatoma cells) for 2 h. The beads were suspended in 100 µl of NET after three washes with NET-2 buffer (500 µl each time), 50 µl of 2 M sodium acetate containing 500 µg/ml carrier tRNA and 350 µl of 8 M guanidine HCl in 50 mM Tris-HCl, pH 7.5, 10 mM EDTA. The samples were mixed well and centrifuged for 5 min to remove Sepharose beads. RNA was extracted with 300 µl of chilled absolute ethanol as described (24) and subjected to electrophoresis on 10% polyacrylamide, 7 M urea gels and detected by autoradiography.

RESULTS AND DISCUSSION

Antibodies Raised against Poly(A) Polymerase from Morris Hepatoma 3924A React with the Corresponding Enzyme from Hepatoma 7777 Cells, but Not with the Enzyme from the Cell Line H4—Previous studies in our laboratory have demonstrated that nuclear poly(A) polymerase from Morris hepatoma 3924A (a transplanted solid tumor) is structurally distinct from the corresponding rat liver enzyme and that antibodies raised against the hepatoma enzyme do not react with the liver enzyme. It was, therefore, crucial to determine whether the anti-hepatoma poly(A) polymerase antibodies will react with the enzyme from the 7777 hepatoma cell line used for labeling the RNA but not with the enzyme from a cell line that exhibits properties of normal hepatocytes (25).

To test this possibility, the proteins in the nuclear extracts from the cell line H4 and hepatoma 7777 cells were separated on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose paper, and probed with antisera raised against hepatoma 3924A poly(A) polymerase (for details of the immuno- blot analysis, see "Materials and Methods"). The cell line derived from another Morris hepatoma instead of the hepatoma 3924A was selected for the present studies, as a suitable cell line from the latter solid tumor was not available. The nuclear extracts from the hepatoma 7777 cells (Fig. 1, lane 2) formed a distinct immune complex corresponding to a molecular weight of 48,000. A similar complex was also formed with highly purified poly(A) polymerase from hepatoma 3924A used as the control (lane 3). Under these conditions, none of the proteins from the cell line H4 reacted with the antibodies (lane 1). The preimmune control sera did not react with either tumor enzyme (lanes 4–6). These data demonstrated the specificity of the anti-poly(A) polymerase antibodies and allowed their use in immunoprecipitation using the hepatoma 7777 cell line.

Anti-poly(A) Polymerase Antibodies Precipitate U1 RNA—Previous studies in other laboratories have indirectly suggested a potential role of small nuclear RNAs (26) in the mRNA polyadenylation (see the Introduction). The availability of specific antibodies against tumor-type poly(A) polymerase prompted us to investigate whether these antibodies can immunoprecipitate one or more of the snRNAs. We reasoned that if poly(A) polymerase is tightly bound to snRNA(s) as a functional polyadenylation complex, the poly(A) polymerase antibodies should form an immunocomplex with the snRNA and/or its associated protein(s). For this purpose, the hepatoma 7777 cells were labeled with [32P]phosphate followed by

![Fig. 1. Immunoblot analysis of poly(A) polymerase from hepatoma 7777 cells and the cell line H4. Nuclear extracts from H4 cells (lanes 1 and 4), hepatoma 7777 cells (lanes 2 and 5), highly purified tumor-type poly(A) polymerase (lanes 3 and 6), and marker proteins (lane M) were fractionated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose paper, and probed with either anti-hepatoma poly(A) polymerase antisera (lanes 1, 2, and 3) or preimmune sera (lanes 4, 5, and 6) as described under "Materials and Methods." The arrow indicates the position of poly(A) polymerase corresponding to a molecular mass of 48,000 daltons. M corresponds to marker proteins stained with Amido Black (a = 97,000; b = 67,000; c = 43,000; d = 30,000; e = 20,000; and f = 14,000 daltons).](image-url)
incubation with the antibodies raised against purified poly(A) polymerase from the hepatoma 3924A. RNA was extracted and analyzed on a high percentage gel to visualize only the low molecular weight RNAs as described under "Materials and Methods." Only one 32P-labeled band corresponding to a size of 172 nucleotides was precipitated (Fig. 2, lane 2) whereas no detectable RNA was observed when preimmune sera were used for precipitation (lane 3). To determine the nature of the RNA associated with poly(A) polymerase, human RNP antisera that are known to react only with U1 snRNA (27) were used as a control. As anticipated, the human sera precipitated only U1 RNA which corresponded exactly to the RNA contained in the immune complex with anti-poly(A) polymerase antibodies (lane 1). Since all snRNAs contain a 5' cap (m7G) structure, antibodies against this structure should immunoprecipitate the RNA associated with poly(A) polymerase. To test this possibility, the nuclear extract from 32P-labeled McA-RH-7777 cells was reacted with trimethyl cap sera. The major RNA immunoprecipitated with this sera corresponded exactly to the RNA immunoprecipitated with anti-poly(A) polymerase antibodies (lane 5).

Antibodies against Hepatoma Poly(A) Polymerase Do Not Immunoprecipitate U1 RNA from a Cell Line Exhibiting Properties of Hepatocytes—To determine the specificity of the antibodies against the tumor-type enzyme, H4 cells which do not contain tumor-type enzyme (see Fig. 1) were used for labeling the RNA and immunoprecipitation. If U1 RNA from the H4 cell line is associated with the liver-type poly(A) polymerase, antibodies against the tumor enzyme should not precipitate U1 RNA from this cell line. No detectable RNA was observed following analysis of the immunoprecipitate whereas the anti-RNP sera precipitated U1 RNA (Fig. 3, lane 2). Another sera, human Sm antisera that are known to react with U1, U2, U4, U5, and U6 snRNAs precipitated all these RNAs from H4 cells (lane 1). Preimmune sera used as an additional control (lane 3) did not react with snRNAs from the H4 cells. These observations suggest that immunoprecipitation of U1 RNA with anti-tumor poly(A) polymerase antibodies (see Fig. 2) is a specific reaction.

Anti-RNP Antisera Do Not React with Poly(A) Polymerase—Because an anti-poly(A) polymerase antisera can react with U1 RNA, it was of interest to know whether anti-RNP antisera can react with poly(A) polymerase. Such an experiment will eliminate the possibility of cross-reactivity of anti-poly(A) polymerase antibodies with U1 RNA or U1 RNP. This issue was addressed by probing the hepatoma 7777 nuclear extract or purified poly(A) polymerase protein blots with human anti-RNP antisera or normal human sera (Fig. 4). Neither sera reacted with poly(A) polymerase (Fig. 4, lanes 1 and 4).
were fractionated on tomato. The nitrocellulose blots were probed with anti-RNP antisera and corresponds to markers 20,000; and the RNA, determined by plotting distance migrated in millimeters versus log (nucleotides) of φX174 DNA markers is 172 nucleotides which corresponds to the length of Ula RNA (27). Although cleavage of RNAs does not appear to inhibit addition of poly(A) to mRNA (14, 15), the lack of complete degradation of the snRNA under these conditions does not preclude the potential role of any one of these molecules in the polyadenylation reaction (18). The association of U-type snRNP with the 50 S polyadenylation complex, identified by density gradient fractionation (28), is consistent with a possible involvement of snRNA in mRNA polyadenylation. Although our studies have not directly demonstrated the role of U1a snRNA in the 3' end processing of adenovirus L3 mRNA, the specific association of this RNA with poly(A) polymerase suggests that it is involved in some aspect of cleavage/polyadenylation. Since anti-poly(A) polymerase antibodies can inhibit polyadenylation of adenovirus L3 mRNA,2 U1a RNA is probably associated with a functional poly(A) polymerase in the polyadenylation complex.

FIG. 4. Immunoblot analysis of nuclear extract from hepatoma 7777 cells and purified poly(A) polymerase using anti-RNP antibodies. Nuclear extract from hepatoma 7777 cells (lanes 1 and 2) and purified tumor-type poly(A) polymerase (lanes 3 and 4) were fractionated on 10% SDS-polyacrylamide gel and transferred to nitrocellulose paper as described under “Materials and Methods.” The nitrocellulose blots were probed with anti-RNP antisera (lanes 1 and 2) or with normal human sera (lanes 3 and 4). Lane M corresponds to markers (a = 97,000; b = 67,000; c = 43,000; d = 20,000; and e = 14,000 daltons).

REFERENCES
1. Darnell, J. E. (1982) Nature 297, 365-371
2. Nevins, J. R. (1983) Annu. Rev. Biochem. 52, 441-466
3. Birnstiel, M. L., Busslinger, M., and Struf, K. (1985) Cell 45, 349-359
4. Proudfoot, N. J., and Whitelaw, E. (1988) in Frontiers in Molecular Biology—Transcription and Splicing (Glover, D. M., and Haines, B. D., eds) IRL Press, Oxford, in press
5. Leff, F. W., Rosenfeld, M. G., and Evans, R. M. (1986) Annu. Rev. Biochem. 55, 109-117
6. Zarkower, D., and Wickens, M. (1987) EMBO J. 6, 4185-4192
7. Proudfoot, N. J., and Brownlee, G. G. (1976) Nature 263, 211-214
8. Hashimoto, C., and Steitz, J. A. (1986) Cell 45, 581-591
9. Zhang, F., and Cole, C. N. (1987) Mol. Cell. Biol. 7, 3277-3286
10. Skolnik-David, H., Moore, C. L., and Sharp, P. A. (1987) Genes and Dev. 11, 672-682
11. Zarkower, D., and Wickens, M. (1987) EMBO J. 6, 177-186
12. Humphrey, T., Christoffori, G., Lucyanic, V., and Keller, W. (1987) EMBO J. 6, 4159-4168
13. Conway, L., and Wickens, M. (1987) EMBO J. 6, 4177-4184
14. Ryner, L. C., and Manley, J. L. (1987) Mol. Cell. Biol. 7, 495-503
15. Berget, S. M., and Robberson, B. L. (1986) Cell 46, 691-695
16. Rose, K. M., and Jacob, S. T. (1976) Eur. J. Biochem. 67, 11-21
17. Rose, K. M., Allen, M. S., Crawford, I. L., and Jacob, S. T. (1978) Eur. J. Biochem. 88, 29-36
18. Rose, K. M., and Jacob, S. T. (1979) J. Biol. Chem. 254, 10256-10261
19. Stetler, D. A., and Jacob, S. T. (1984) J. Biol. Chem. 259, 7239-7244
20. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475-1488
21. Lamml, U. K. (1970) Nature 227, 680-685
22. Minori, T., Hinterferger, M., Petterson, I., and Steitz, J. A. (1984) J. Biol. Chem. 259, 560-565
23. Petterson, I., Hinterferger, M., Minori, T., Gottlieb, E., and Steitz, J. A. (1984) J. Biol. Chem. 259, 5907-5914
24. Cox, R. A. (1968) Methods Enzymol. 12, 120-129
25. Kelley, D. S., Becker, J. E., and Potter, V. R. (1978) Cancer Res. 38, 4591-4600
26. Moore, C. L., and Sharp, P. A. (1984) Cell 36, 581-591
27. Lerner, M. R., and Steitz, J. A. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5495-5499
28. Moore, C. L., Skolnik-David, H., and Sharp, P. A. (1988) Mol. Cell. Biol. 8, 226-233