Association of Newly Synthesized Poly(A) Polymerase with Four Distinct Polypeptides*

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Nuclear poly(A) polymerase was isolated from [35S]methionine-labeled hepatoma McA-RH 7777 cells and subjected to DEAE-Sephadex chromatography. Flow-through and low salt wash fractions containing poly(A) polymerase activity were pooled and subjected to immunoblot analysis using anti-tumor type poly(A) polymerase antibodies and a biotinylated second antibody. The immune complex contained a single 48-kDa polypeptide band corresponding to the tumor-type enzyme. When immunoprecipitations were carried out using the same fraction and antibodies, at least five [35S]-methionine-labeled proteins with approximate molecular masses of 74, 48, 35, 30, and 22 kDa were observed. Pulse-chase studies did not indicate a precursor-product relationship between the immunoprecipitated proteins. Preimmune sera did not react with poly(A) polymerase or other components in the protein complex. These data show that poly(A) polymerase exists as part of a complex with at least four other polypeptides and suggest that these polypeptides may be involved in the cleavage and/or polyadenylation reactions.

Poly(A) polymerase is the enzyme believed to catalyze the post-transcriptional addition of a poly(A) tract to the 3' end of mRNAs. Bacterial poly(A) polymerase (EC 2.7.7.19) is present in a wide variety of tissues and cells (1, 2). Poly(A) polymerase is predominantly a nuclear enzyme, but it is also located in various organelles such as the mitochondria (3), ribosomes (4), and the cytosol (5). For many years, our laboratory has studied the structural and immunological characteristics of nuclear poly(A) polymerase (6, 7). Nuclear poly(A) polymerase isolated from normal rat liver is both structurally and immunologically distinct from the corresponding enzyme isolated from Morris hepatoma 3924A (8). Poly(A)* RNA from the hepatoma can be translated to tumor-type poly(A) polymerase whereas no corresponding product is produced from liver poly(A)* RNA using a rabbit reticulocyte system (9), which indicates that a functional mRNA for tumor-type poly(A) polymerase is absent from adult rat liver.

We have now turned our attention to characterizing the role of poly(A) polymerase in the polyadenylation reaction. The two steps involved in the 3' end processing of pre-mRNA are the endonucleolytic cleavage of the precursor RNA and the subsequent addition of poly(A) tract at the cleavage site. Two sequences within the mRNA precursor are essential for efficient cleavage and polyadenylation. These are the highly conserved AUUAAA or a related sequence found 5-30 nucleotides upstream of the polyadenylation site (10-12) and the less well conserved downstream element (13-15).

In vitro polyadenylation systems have been developed that allow efficient cleavage and polyadenylation of exogenously added mRNA precursors (16, 17). Results from studies utilizing in vitro polyadenylation systems suggest that one or more factors in the extracts may be interacting with the cis-acting sequences in pre-mRNAs. Studies in several laboratories have suggested that cleavage/polyadenylation occurs in a complex (18-25), but the components in this complex have not been fully characterized.

The availability of highly specific antibodies against the tumor-type poly(A) polymerase (29) prompted us to explore the role of this enzyme in mRNA polyadenylation. Recently, we have used these antibodies to demonstrate the close association of U1 RNA with poly(A) polymerase (30). The present studies were directed to examine whether poly(A) polymerase synthesized de novo is associated with other polypeptides as part of a complex.

MATERIALS AND METHODS

[35S]Methionine > 800 Ci/mmol was purchased from Du Pont-New England Nuclear and Pansorbin from Calbiochem. All other reagents were of high quality grade.

Cell Maintenance and Labeling—McA-RH 7777 cells were initially provided by Dr. V. R. Potter (26) and subsequently maintained in modified Swim's S77 medium as described (27). Cells used in labeling procedures were grown to 80-100% confluence (approximately 1 × 10^7 cells/100-mm plate), washed twice in phosphate-buffered saline (0.5 M sodium phosphate, pH 7.5, 150 mM NaCl), and starved for 2 h at 37 °C prior to labeling in methionine-free Dulbecco's minimal essential medium. For continuous labeling procedures, cells were maintained in methionine-free minimal essential medium with 25 μCi/ml [35S]methionine for 2 h at 37 °C prior to harvest. For pulse-chase labeling, cells were starved and labeled as described for continuous labelings, then washed twice with methionine-containing medium (S77), and maintained for 2 h at 37 °C in S77 prior to harvest.

Harvesting of Cells and Preparation of Nuclear Extract—Cells were harvested by centrifugation (10 min, 1033 g), and the cell pellet was washed twice with phosphate-buffered saline. Nuclear extract was prepared essentially by the method of Dignam et al. (28). Washed McA-RH 7777 cells were resuspended in three packed cell volumes of buffer A (10 mM Hepes, 1 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) and allowed to stand on ice for 10 min. The cells were centrifuged (10 min, 1033 g), resuspended in three packed cell volumes of buffer B (20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 0.5 mM DTT), and allowed to stand on ice for 10 min. The cells were centrifuged (10 min, 1033 g), resuspended in three packed cell volumes of buffer A, and lysed by homogenization with a Wheaton glass Dounce homogenizer (type B pestle). The homogenate was centrifuged to obtain the crude nuclear pellet. The crude nuclear pellet was resuspended (20 min, 33,000 g), resuspended in 1.5 packed cell volumes of buffer B (20 mM Hepes, pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT), stirred for 30 min at 4 °C, and centrifuged (30 min, 33,000 g). The supernatant was dialyzed for 2 h against 50 volumes of buffer C (20 mM Hepes, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged (20 min, 19270-19273 1988
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25,000 x g). The supernatant was precipitated with solid (NH₄)₂SO₄ (0.472 g/ml), resuspended, and dialyzed against buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride). The protein content of the nuclear extract was estimated using the Pierce BCA protein assay reagent.

DEAE-Sephadex Column Chromatography—The nuclear extract was fractionated on a DEAE-Sephadex (A-25) column (1-ml bed volume/mg of protein) equilibrated in buffer E (50 mM Tris-HCl, pH 7.9, 25% glycerol, 5.0 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT). The column was eluted stepwise with buffer E containing 20, 50, 100, and 400 mM (NH₄)₂SO₄, respectively. The poly(A) polymerase fraction was represented by the combined flow-through and 20 mM wash fractions. All fractions were dialyzed against 50 mM Tris-HCl, pH 7.9, 20% glycerol, 0.5 mM DTT and stored at -40 °C until further use.

Assay for Poly(A) Polymerase Activity—Fractions were assayed for poly(A) polymerase activity as described (7).

Immunoprecipitation—The antibodies used for immunoprecipitation were prepared essentially according to a previously described protocol (29). Briefly, a male New Zealand White rabbit was injected biweekly with 50 μg of highly purified tumor type nuclear poly(A) polymerase plus Freund's complete adjuvant. Sera were collected after immunization and purified by chromatography on a DEAE-Affi-Gel blue column (Bio-Rad). Sera collected prior to immunization were used as control (preimmune) sera. Generally, partially purified poly(A) polymerase from McA-RH 7777 cells corresponding to 1 x 10⁶ cpm in a standard enzyme assay was used for each immunoprecipitation reaction performed as described (9).

Purification of Poly(A) Polymerase from Morris Hepatoma 3924A—Poly(A) polymerase was purified from Morris hepatoma 3924A nuclear extract as described (7). It consisted of sequential chromatography on DEAE-Sephadex, QAE-Sephadex, phosphocellulose, hydroxylapatite, and DNA cellulose.

Immunoblot Analysis—Purified poly(A) polymerase from Morris hepatoma 3924A and partially purified enzyme from hepatoma McA-RH 7777 cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the polypeptides were transferred to nitrocellulose paper (Schleicher and Schuell) at 100 mA for 10 h. The blot was incubated for 2 h at 20 °C with biotinylated anti-rabbit IgG (Jackson ImmunoResearch). When the nitrocellulose blots were reacted with anti-tumor-type poly(A) polymerase antibodies, a single band of molecular mass of 48 kDa (lanes 3 and 5) was observed with poly(A) polymerase preparations from both tumor. This 48-kDa band was not observed when control sera were used. We have previously reported the molecular mass of tumor type poly(A) polymerase to be 48 kDa (8). The results of the immunoblot analysis demonstrate that (a) the antibody for tumor-type poly(A) polymerase is specific and (b) tumor-type poly(A) polymerase is present in McA-RH 7777 cells.

Association of Poly(A) Polymerase with Other Polypeptides—To examine de novo synthesis of tumor type poly(A) polymerase, hepatoma McA-RH 7777 cells were labeled for 2 h with [35S]methionine, and nuclear poly(A) polymerase was isolated and partially purified by DEAE-Sephadex chromatography. Poly(A) polymerase was recovered from the flow-through and first 20 mM (NH₄)₂SO₄ wash, and immunoprecipitations were carried out using IgG purified from anti-tumor-type poly(A) polymerase antisera or control sera (see "Materials and Methods"). Fig. 2A shows the results of immunoprecipitation reactions utilizing these fractions. Under the same exposure conditions used for Fig. 2A, only a light 48-kDa poly(A) polymerase band and the associated polypeptides were observed in the second 20 mM wash fraction. The film was therefore exposed for significantly longer periods to detect the polypeptides. By doing so, a similar profile of polypeptides was detected in the second 20 mM wash fraction (Fig. 2B, lane 1) and to some extent in the 50 mM wash fraction (Fig. 2B, lane 3), whereas the 100 and 400 mM salt wash (lanes 5 and 7) had no

FIG. 1. Immunoblot of poly(A) polymerase from Morris hepatoma 3924A and McA-RH 7777 cells. Ten micrograms of highly purified poly(A) polymerase from Morris hepatoma 3924A (lanes 2 and 4) and partially purified poly(A) polymerase from McA-RH 7777 cells (lanes 3 and 5) were subjected to electrophoresis and transferred to nitrocellulose paper as described under "Materials and Methods." Proteins were reacted with immune sera (lanes 2 and 3) and control (preimmune) sera (lanes 4 and 5). Molecular mass markers in lane 1 are as follows: 94.0 kDa, phosphorylase b; 67.0 kDa, bovine serum albumin; 43.0 kDa, ovalbumin; 30.0 kDa, carbonic anhydrase; and 20.1 kDa, soybean trypsin inhibitor.

FIG. 2. Co-precipitation of additional newly synthesized polypeptides with anti-poly(A) polymerase antibodies suggest close association of poly(A) polymerase with other proteins in a complex.

To investigate further the association of poly(A) polymerase synthesized de novo with other polypeptides, the DEAE-Sephadex column was eluted stepwise with a second 20 mM wash followed by elution with 50, 100, and 400 mM (NH₄)₂SO₄. Fig. 2B shows the results of immunoprecipitation reactions utilizing these fractions. Under the same exposure conditions used for Fig. 2A, only a light 48-kDa poly(A) polymerase band and the associated polypeptides were observed in the second 20 mM wash fraction. The film was therefore exposed for significantly longer periods to detect the polypeptides. By doing so, a similar profile of polypeptides was detected in the second 20 mM wash fraction (Fig. 2B, lane 1) and to some extent in the 50 mM wash fraction (Fig. 2B, lane 3), whereas the 100 and 400 mM salt wash (lanes 5 and 7) had no
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FIG. 2. Immunoprecipitation of partially purified poly(A) polymerase from McA-RH 7777 cell nuclear extract. A, McA-RH 7777 cells were labeled as described under "Materials and Methods." Poly(A) polymerase was partially purified from 7777 cell nuclear extract by DEAE-Sephadex chromatography, immunoprecipitated with anti-tumor type poly(A) polymerase antibodies, and subjected to polyacrylamide gel electrophoresis under denaturing conditions. 

polymerase activity was also eluted in the flow-through and the first 20 mM wash fractions (data not shown), which was consistent with our previous observations (8). It should be emphasized that no polypeptides other than those detected in the flow-through and the first 20 mM wash fractions were observed in any fraction and that preimmune sera did not immunoprecipitate any corresponding polypeptides.

Poly(A) Polymerase-associated Polypeptides Are Not Cleavage Products from a Large Precursor—Pulse-chase studies were undertaken to examine the possibility that precursor-product relationships may exist for the [35S]methionine-labeled proteins of the immunoprecipitated complex. McA-RH 7777 cells were labeled with [35S]methionine for 2 h and chased for 2 h in the presence of excess unlabeled methionine as described under "Materials and Methods." Poly(A) polymerase was isolated and partially purified by DEAE-Sephadex chromatography, and immunoprecipitations were carried out as before. The pulse-chase experiment (Fig. 3, compare lanes 1 or 2 with lane 4) showed that the radioactivity in the 74/72-kDa bands decreased markedly whereas there was only a slight increase in the intensity of other bands. The increased radioactivity in the 48-22-kDa bands was not proportional to the virtual disappearance of the labeled 74/72-kDa band from the autoradiogram. Further, an additional 4-h (total 6 h) chase did not increase the radioactivity in the 48-22-kDa polypeptides (data not shown). A minor 40-kDa protein band observed after pulse-chase (lane 4) may be derived from the 74/72-kDa protein. Overall, these data do not indicate a precursor-product relationship between the high molecular weight polypeptide and other labeled bands in the autoradiogram. The rapid disappearance of the radioactivity in the band corresponding to 74/72 kDa suggests that this protein may have a short half-life. The specificity of the antibodies used in this experiment was further evident from the absence of any major labeled proteins after immunoprecipitation with control sera (Fig. 3, lane 3).

The existence of a complex involved in 3' end processing of pre-mRNA has been proposed by several investigators (for a review, see Ref. 31). When in vitro polyadenylation reactions containing four different pre-mRNA substrates were probed with RNase T1 and antibodies specific for Sm small nuclear ribonucleoproteins, RNA fragments containing the AAUAAA signal were immunoprecipitated (19). A complex between components of the HeLa cell nuclear extract and thymidine kinase (22) or adenovirus precursor RNA (23, 25) has been identified using gel mobility shift analysis. The complex formation was markedly reduced with substrates lacking a pol-
yadenylation signal (22) or by point mutation in the hexanucleotide sequence (25) or by the downstream deletion mutation (23). More recent studies (24) have shown by glycerol gradient sedimentation analysis that a 50 S complex intermediate is formed during the polyadenylation reaction. Although this complex contains U-type small nuclear ribonucleoprotein particles their functional significance is unclear. Similarly, other components in this complex have not been characterized with respect to their functions and properties.

We have taken a different approach which employed antibodies raised against purified poly(A) polymerase. The results of these studies are summarized in Fig. 4. The data indicate that poly(A) polymerase synthesized de novo is associated with at least four other polypeptides. It is unlikely that immunoprecipitation of 35S-labeled polypeptides with IgG purified from immune sera is caused by contaminant antibodies in the sera. First, purified enzyme preparation used for raising the antibodies contained a single protein corresponding to 48 kDa (see Ref. 8). Second, the antigen was frequently electroeluted following sodium dodecyl sulfate-polyacrylamide gel electrophoresis prior to immunization. Third, only those fractions containing poly(A) polymerase activity immunoprecipitated the additional polypeptides (Fig. 2A, lane 1; Fig. 2B, lane 1). Fourth, only one band corresponding to the 48-kDa poly(A) polymerase was observed after immunoblotting of relatively crude nuclear extracts. Further, the inability of control sera to precipitate 35S-labeled polypeptides rules out the possibility of nonspecific interaction of the antibody with other polypeptides. Pulse-chase experiments do not reveal any significant precursor-product relationship between the polypeptides. These data, taken together, indicate that poly(A) polymerase exists in vivo as a complex with other proteins. Although the functions of the other polypeptides are not evident from these studies, at least one of them could be the endonuclease that selectively cleaves pre-mRNA at the poly(A) site.

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