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Cell-Free Synthesis of Tumor-Type Poly(A) Polymerase†

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ABSTRACT: Previous studies in this laboratory suggested that in adult liver, either the gene for the tumor-type poly(A) polymerase is poorly transcribed or the mRNA for this enzyme is largely not expressed. To test these possibilities, total RNA from rat liver and Morris hepatoma 3924A RNA were isolated by using a guanidine thiocyanate method; poly(A+) RNA and poly(A−) RNA were separated by oligo(dT)–cellulose chromatography and used for translation in a rabbit reticulocyte lysate system. After in vitro translation, the products were immunoprecipitated with either purified anti-tumor poly(A) polymerase antibodies or control immunoglobulins. When the polypeptides translated from poly(A+) or poly(A−) hepatoma RNA were precipitated with immune sera, a unique [^{35}S]methionine-labeled 35-kilodalton (kDa) protein was observed. This band was not apparent when control serum was used for the immunoprecipitation. The radiolabeled 35-kDa polypeptide was not evident when the products were incubated with highly purified tumor nuclear poly(A) polymerase prior to immunoprecipitation. Prior incubation of the translation products with bovine serum albumin instead of poly(A) polymerase had no effect on the immunoprecipitation. This 35-kDa protein was not apparent when liver poly(A+) RNA was used to direct translation. These data demonstrate that (a) the tumor enzyme is not synthesized as a precursor, (b) tumor mRNA, but not normal liver mRNA, contains detectable sequences coding for tumor-type poly(A) polymerase, and (c) poly(A) polymerase mRNA also exists as a poly(A−) population.

Eukaryotic poly(A) polymerase (EC 2.7.7.19) is a ubiquitous enzyme present in a great variety of cells [for reviews, see Jacob & Rose (1983) and Edmonds (1982)]. The major function of the enzyme appears to be the posttranscriptional addition of a poly(A) tract to the 3′ termini of mRNAs. Although poly(A) polymerase is largely localized in the nucleus, it is also present in the mitochondria (Jacob et al., 1972), microsomes (Wilkie & Smellie, 1968), ribosomes (Milchev et al., 1980), and cytosol (Tsiapalis et al., 1975) fractions. Over the years, our laboratory has been studying the structure, function, and posttranslational modifications of nuclear poly(A) polymerase (Rose et al., 1977, 1978; Rose & Jacob, 1979, 1980).

Recent studies have shown that the major nuclear poly(A) polymerase from normal liver is structurally and immunologically distinct from the corresponding enzyme of transplanted (Stetler & Jacob, 1984) and of azo dye induced primary hepatomas (Stetler et al., 1984). Polyclonal antibodies raised against hepatoma nuclear poly(A) polymerase reacted specifically with the tumor enzyme, but not with the liver enzyme (Stetler & Jacob, 1984). These studies have shown that the difference in the molecular weights of the tumor and liver nuclear poly(A) polymerase is not due to proteolytic

† This work was supported by U.S. Public Health Service Grants CA 25078 and CA 31894 from the National Cancer Institute to S.T.J.

0006-2960/86/0425-1515$01.50/0

cleavage of the "tumor-type" poly(A) polymerase to the "liver-type" enzyme but that they represent structurally distinct entities. To prove further that the two enzymes are products of two separate genes and to determine whether poly(A) polymerase is synthesized as a precursor, poly(A+) and poly(A−) mRNAs isolated from Morris hepatoma 3924A and normal rat liver were translated in a cell-free system followed by immunoprecipitation of poly(A) polymerase with antibodies raised against the hepatoma enzyme.

Experimental Procedures

Materials. Oligo(dT)–cellulose type 2 was a product of Collaborative Research, Inc. (Lexington, MA). Rabbit globin mRNA was purchased from Bethesda Research Laboratories, Inc. (Rockville, MD). [^{35}S]Methionine (1030–1460 Ci/mmol) was obtained from Amersham Corp. (Arlington, IL). Micrococcal nuclease and calf liver tRNA were products of Boehringer Mannheim Biochemicals (Indianapolis, IN). Pansorbin was purchased from Calbiochem-Behring (La Jolla, CA). All other reagents were of high-quality grade.

Isolation of Poly(A+) RNA from Hepatoma and Liver. Total RNA was isolated by a combination of two protocols (Cathala et al., 1983; Maniatis et al., 1982). Male rats (ACI strain) with transplanted Morris hepatoma 3924A were sacrificed by decapitation; the tumors were removed quickly, minced, and homogenized (20-g batches) with a Polytron blender in buffer containing 4 M guanidine thiocyanate. RNA was precipitated with 4 M LiCl, extracted with sodium dodecyl sulfate–phenol, and precipitated at −20 °C for 16 h by the addition of 0.3 M sodium acetate and 2 volumes of cold ethanol. Poly(A+) RNA and poly(A−) RNA were selected by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972; Maniatis et al., 1982). RNA that was unbound to the oligo(dT)–cellulose column was reapplied to the column, and the fraction which did not bind to the column at this stage was used as poly(A−) RNA. Poly(A+) RNA was eluted with 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] pH 7.5, and 1 mM EDTA. RNA was precipitated as described above. Poly(A+) RNA from normal (ACI strain) rat liver was isolated by the same method.

Preparation of Rabbit Reticulocyte Lysate. The lysate was prepared essentially by the method of Pelham and Jackson (Pelham & Jackson, 1976; Jackson & Hunt, 1983). The reaction mixture for preparing messenger-dependent lysate contained lysate, 25 μM hemin, 50 μg/mL creatine kinase, 1 mM calcium acetate, and 150 units/mL micrococcal nuclease and was incubated for 20 min at 20 °C. The digestion was stopped by the addition of 2.0 mM EGTA. In some cases, 60 μg/mL of calf liver tRNA (Boehringer Mannheim) was added to the lysate which was then quick-frozen and stored at −70 °C in aliquots until further use.

Cell-Free Translation. The reaction mixture (total volume 20 μL) contained 100 mM potassium chloride, 0.5 mM magnesium chloride, 10 mM creatine phosphate, 50 μM each of 19 unlabeled amino acids, 0.5 mM DTT, 15–30 μCi of [^{35}S]methionine, 70–500 μg/mL RNA, and 17–20 μL of messenger-dependent rabbit reticulocyte lysate. Unless otherwise indicated, the incubations were carried out at 30 °C for 80 min with gentle shaking. After incubation, the radioactivity incorporated into protein was determined by mixing 2–5-μL samples with 0.5 mL of 5% hydrogen peroxide in 1 N sodium hydroxide and incubating for 10 min at 37 °C followed by the addition of 3.0 mL of 2% L-methionine in 25% trichloroacetic acid. The samples were filtered through GF/C filters and counted in an Isocap/300 liquid scintillation system using 5.0 mL of 949 premixed scintillation fluor (New England Nuclear).

Immunoprecipitation and Polyacrylamide Gel Electrophoresis. The antibodies used for the immunoprecipitation were prepared as described (Jacob & Rose, 1983b). Briefly, male New Zealand White rabbits were injected biweekly with 50 μg of high purified tumor nuclear poly(A) polymerase combined with an equal volume of Freund's complete adjuvant. Rabbit sera were prepared from whole blood, and in some cases, the IgG fraction was isolated by chromatography on a DEAE-Affi Gel Blue column as described by the manufacturer (Bio-Rad Laboratories). Serum from a rabbit prior to immunization (preimmune sera) or after immunization with poly(A) polymerase (immune sera) was used for the analysis of the translation product. After in vitro translation, samples were pooled, 2-μL aliquots were used for radioactivity measurements, and the remainder was used for immunoprecipitation. In general, 500 000 cpm were used for the immunoprecipitation. The lysate was diluted to 95 μL with a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 5 mM EDTA, 0.1 mM PMSF, and 0.05% NP-40 and preabsorbed with crude rabbit serum (5 μL) for 1 h at 20 °C and then for 16 h at 4 °C. Following preabsorption, 50 μL of Pansorbin (BRL) was added to the mixture and incubated for 1–2 h at 20 °C. Samples were centrifuged at 12000*g* for 5 min and supernatant fractions collected. To the supernatant was added either 5 μL (unless otherwise specified in the figure legends) of purified control or 5 μL of anti-poly(A) polymerase IgG; incubation continued for 1 h at 20 °C followed by 16 h at 4 °C. Fifty microliters of Pansorbin (BRL) was then added to the mixture and incubated as above. Following centrifugation, the pellet was saved and washed 3 times with buffer containing 50 mM potassium phosphate (pH 7.5), 100 mM sodium chloride, 1% Triton X-100, 1% deoxycholate, and 0.1% sodium dodecyl sulfate, washed 2 times with buffer containing 150 mM sodium phosphate, pH 7.4, and 150 mM sodium chloride, resuspended in 62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate, and 5% (v/v) β-mercaptoethanol, and subjected to polyacrylamide electrophoresis under denaturing conditions according to Laemmli (1970). The following molecular weight markers were used for determination of the immunoprecipitated product size: 200 kDa, myosin; 130 kDa, β-galactosidase; 97.4 kDa, phosphorylase *b*; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor, and 14.4 kDa, lysozyme. Following electrophoresis, the proteins were fixed with 2-propanol and acetic acid, stained with Coomassie Brillant Blue R-250, and treated with EN^{3}HANCE (New England Nuclear) according to the manufacturer's specifications. The protein bands were visualized by autoradiography using Kodak XAR film and an intensifying screen.

Immunoblotting. Nuclear poly(A) polymerase was purified from Morris hepatoma 3924A and normal rat liver as described previously (Rose et al., 1978; Rose & Jacob, 1976; Stetler & Jacob, 1985a). The enzyme thus prepared was essentially homogeneous as demonstrated by a single Coomassie Blue stained band following SDS–PAGE.

Partially purified poly(A) polymerase preparations from Morris hepatoma 3924A and rat liver were subjected to po-

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s); DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

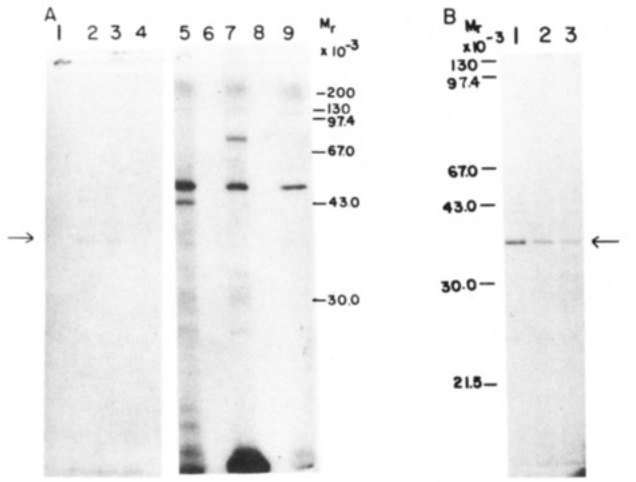

FIGURE 1: Autoradiogram of rat hepatoma poly(A+) RNA directed translation products. (A) The concentration of RNA was 72 μg/mL. Immune sera dilution for lanes 1–4 was 1:1600. Lanes 1 and 2 correspond to translation products precipitated with control sera and immune sera, respectively. Lanes 3 and 4 represent autoradiograms of translation products precipitated with immune sera after addition of 25 μg (lane 3) or 50 μg of purified poly(A) polymerase. Lane 5 corresponds to total translation products not immunoprecipitated. Controls included immunoprecipitate derived from globin mRNA directed translation (lane 6), nonimmunoprecipitated globin mRNA directed translation products (lane 7), and total products derived from samples incubated without RNA and then immunoprecipitated (lane 8) or not immunoprecipitated (lane 9). (B) The dilution of immune sera used for immunoprecipitation was 1:400. Lane 1 corresponds to translation products precipitated with immune sera; lanes 2 and 3 represent precipitated products after addition of 25 μg (lane 2) or 50 μg (lane 3) of purified poly(A) polymerase. The molecular weight markers shown in (A) and (B) are as described under Experimental Procedures. The arrow corresponds to the 35-kDa polypeptide.

lyacrylamide gel electrophoresis under denaturing conditions. After electrophoresis, the gel was incubated in a buffer containing 100 mM Tris-HCl, pH 7.0, 50 mM sodium chloride, 20 mM EDTA, 0.1 mM DTT, and 4 M urea for 2 h. Proteins were transferred to nitrocellulose paper (Schleicher & Schuell) by diffusion as described (Bowen et al., 1980). After transfer, the filter was incubated for 16 h at 37 °C in buffer A [10 mM Tris-HCl, pH 7.5, and 0.9% (w/v) sodium chloride] containing 1% bovine serum albumin followed by incubation with either purified anti-poly(A) polymerase IgG or control IgG at a 1:200 dilution in buffer A containing 5% BSA for 90 min at 20 °C followed by 16 h at 4 °C. Filters were then washed with buffer A and incubated for 1–2 h at 20 °C with ^{125}I-labeled protein A (~500 000 cpm/filter). The paper was then washed extensively in buffer A followed by a detergent wash in 50 mM Tris-HCl, pH 7.4, 1 M sodium chloride, 5 mM EDTA, 0.1 mM PMSF, and 0.4% (w/v) sodium lauryl sarcosinate. After the paper was dried, the filters were subjected to autoradiography as described above.

Results

Cell-Free Translation of Hepatoma Poly(A+) RNA. RNA was isolated from Morris hepatoma 3924A by using guanidine thiocyanate as outlined under Experimental Procedures. The optimal concentration of poly(A+) RNA for translation in a rabbit reticulocyte lysate system was determined for each batch of RNA preparation. Linear incorporation of [^{35}S]methionine into trichloroacetic acid precipitable fractions occurred at a poly(A+) RNA concentration of 70–72 μg/mL (data not presented). Figure 1A represents an autoradiograph of the total translation products and of those obtained after precipitation with preimmune and immune IgG. RNA was used at a concentration of 72 μg/mL. Additional controls included

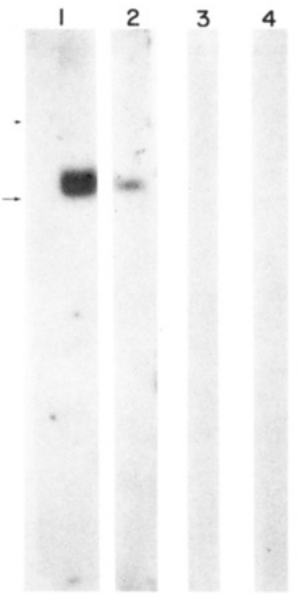

FIGURE 2: Autoradiogram from immunoblot analysis of partially and extensively purified tumor poly(A) polymerase and partially purified liver preparations. Immunoblot analysis was performed as described under Experimental Procedures. Anti-poly(A) polymerase IgG (1:200 dilution) was used to form the immune complex in lanes 1–3. Lanes 1, 2, and 3 correspond to 30 μg of an extensively purified (hydroxylapatite) tumor-type poly(A) polymerase, a partially purified (DEAE-Sephadex) tumor preparation, and a partially purified (DEAE-Sephadex) liver preparation, respectively. Lane 4 is the same as lane 1 except that control IgG replaced anti-poly(A) polymerase IgG. The positions of the molecular weight markers bovine serum albumin (67 kDa) and ovalbumin (43 kDa) are indicated by the upper and lower arrows, respectively.

translation with globin mRNA and samples without RNA. A unique band corresponding to a molecular weight of 35 000 was observed only in the samples immunoprecipitated with anti-poly(A) polymerase antibodies (1:1600 dilution of antibodies).

That the 35-kDa band is indeed poly(A) polymerase was confirmed by adding unlabeled highly purified poly(A) polymerase prior to immunoprecipitation. Thus, when increasing amounts of highly purified tumor poly(A) polymerase were incubated with the translation products prior to immunoprecipitation, no detectable immune precipitate corresponding to the 35-kDa polypeptide was observed (Figure 1A). The specificity of the competition reaction with unlabeled poly(A) polymerase was further proven by using bovine serum albumin (BSA) instead of purified enzyme for competition (data not shown). Even at concentrations of 100 μg per reaction mixture, BSA was not effective in displacing the radioactivity in the immune precipitate corresponding to the 35-kDa band and, therefore, could not replace poly(A) polymerase in the competition reaction.

We then investigated the effect of antibody concentration on the competition reaction (Figure 1B). Lane 1 shows the immune precipitates derived from tumor poly(A+) RNA directed translation using anti-poly(A) polymerase antibodies in a 1:400 dilution. Lanes 2 and 3 correspond to immune precipitates following preincubation with 25 or 50 μg of purified hepatoma poly(A) polymerase, respectively. The intensity of the immunoprecipitated band decreased with increasing amounts of purified enzyme. However, the competition was not as complete as with an antibody dilution of 1:1600.

Further evidence of antibody specificity was provided by the immunoblot analysis (Figure 2). The various enzyme fractions were subjected to polyacrylamide gel electrophoresis

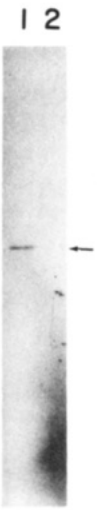

FIGURE 3: Autoradiogram of rat tumor poly(A−) RNA directed translation products. The translation assay contained 500 μg/mL poly(A−) RNA. Lane 1, product after incubation with immune sera; lane 2, immune precipitate obtained after incubation with 105 μg of extensively purified (hydroxylapatite) tumor poly(A) polymerase prior to immunoprecipitation.

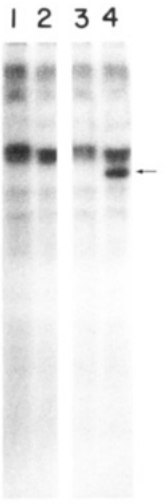

FIGURE 4: Autoradiogram of rat liver poly(A+) RNA directed translation products. The concentration of poly(A+) RNA in the assay was 60 μg/mL for the liver and 72 μg/mL for the tumor RNA. Lanes 1 and 2 are products from liver poly(A+) RNA directed translation, after immunoprecipitation with control sera and immune sera, respectively. Tumor poly(A+) RNA directed translation products are shown in lanes 3 (control sera) and 4 (immune sera).

and subjected to "western blotting" using ^{125}I-protein A as the second antibody as described under Experimental Procedures. Only a single ^{125}I-labeled band was observed after autoradiography. The molecular weight of the antigen in the immune complex obtained with either the partially purified (DEAE-Sephadex) or extensively purified (hydroxylapatite) tumor-type fraction corresponds to that of the authentic protein (Figure 2, lanes 1 and 2). This immune complex is not evident when the partially purified liver fraction is used as the presenting antigen (Figure 2, lane 3) or when control IgG replaces anti-poly(A) polymerase IgG (Figure 2, lane 4).

Cell-Free Translation of Hepatoma Poly(A−) RNA. Poly(A−) RNA, the fraction unbound to the oligo(dT)–cellulose column, was collected after two rounds of chromatography as described under Experimental Procedures. The reaction mixture for cell-free translation was essentially the same as described for the translation of poly(A+) RNA. The polyacrylamide gel electrophoresis profile of poly(A−) RNA directed translation products is shown in Figure 3. Immunoprecipitation of poly(A−) RNA directed translation using anti-poly(A) polymerase antibodies revealed a distinct immune precipitate with an apparent molecular weight of 35 000. To test that this specific band indeed corresponds to tumor poly(A) polymerase, translation products were mixed with tumor poly(A) polymerase prior to immunoprecipitation. Since highly purified enzyme (see Figure 1) or extensively purified enzyme (data not shown) could compete in the immunoprecipitation of the labeled 35-kDa polypeptide following translation of tumor poly(A+) RNA (see Figure 1), we used extensively purified enzyme (purified through DEAE-Sephadex, QAE-Sephadex, phosphocellulose, and hydroxylapatite columns) for competition in this particular experiment. As shown in lane 2, the labeled 35k-kDa polypeptide was not present under these conditions.

The relative absence of poly(A) in the poly(A−) fraction was demonstrated by hybridizing it with poly([5-^{3}H]uridylic acid) (Rosbach & Ford, 1974). The extent of hybridization of poly(A−) RNA (4 μg) to [^{3}H]poly(U) was only 0.2% of that obtained with the same quantity of poly(A+) RNA (data not shown).

Cell-Free Translation of Rat Liver Poly(A+) RNA. Liver RNA was isolated in the same manner as tumor RNA, and poly(A+) mRNA was obtained after oligo(dT)–cellulose chromatography. The reaction mixture for translation was the same as described above, and 60 μg/mL liver poly(A+) mRNA was used in the assay. Figure 4 shows the polyacrylamide gel electrophoresis of translation products obtained after translation of liver poly(A+) mRNA and tumor poly(A+) mRNA. No specific polypeptide was immune precipitated when liver poly(A+) RNA was used for translation whereas the expected 35-kDa tumor enzyme was observed after precipitation with immune sera.

Discussion

These studies have clearly shown that tumor poly(A+) RNA contains translatable sequences for tumor-type poly(A) polymerase and that these sequences are not detectable in poly(A+) RNA from normal liver. These data are consistent with the earlier report from this laboratory (Stetler & Jacob, 1984) that nuclear poly(A) polymerase from the hepatoma is structurally and immunologically distinct from the corresponding liver enzyme. Since fetal liver nuclear poly(A) polymerase is structurally identical with the tumor enzyme (Stetler & Jacob, 1985b), it can be concluded that the relative absence of tumor-type enzyme in normal liver is due to lack of expression of the gene coding for this enzyme in adult liver.

Although poly(A−) mRNA can be translated to poly(A) polymerase, the exact amount of poly(A) polymerase mRNA present in the poly(A−) fraction cannot be determined from these data. Nevertheless, it is conceivable that poly(A−) RNA constitutes a major proportion of poly(A) polymerase mRNA for the following reasons. First, poly(A−) mRNA was obtained after rechromatography of the fraction initially bound to the oligo(dT)–cellulose column. Second, hybridization of the poly(A−) fraction to poly(U) shows negligible levels of poly(A). Third, ribosomal RNA comprises the majority of the RNA in the poly(A) fraction; consequently, the proportion of mRNA in this fraction would be considerably less than that in the poly(A+) fraction.

It should be noted that normal adult rat liver nuclei contain a tumor-type enzyme even though it constitutes only 1% of the nuclear enzyme (Stetler & Jacob, 1984). Recently, the tumor-type enzyme has also been identified in the liver cytosol fraction (Stetler & Jacob, 1985a). The total (nuclear + cy-

tosolic) tumor-type (48-kDa) enzyme in adult liver is only 3% of the total enzyme (Stetler & Jacob, 1985a). Clearly, this relatively low proportion of the tumor-type enzyme is not detectable in the translation product of liver poly(A+) RNA.

These studies have also demonstrated that the tumor-type enzyme is not synthesized as a precursor; in fact, the molecular weight (M_r 35 000) of poly(A) polymerase synthesized in the cell-free system is considerably less than that of purified tumor enzyme (M_r 48 000). Several factors could contribute to the differences in the molecular weight of the in vivo and in vitro products. It is possible that the in vitro translation is incomplete. This could be the result of either premature termination of the peptide chain or rapid proteolytic conversion of the larger product to a smaller entity in vitro. The latter possibility seems unlikely since the rabbit reticulocyte system rarely contains proteolytic enzymes; in fact, this system usually produces a larger precursor of the protein which could be cleaved to functional polypeptide only by exogenously added proteolytic enzymes (Taylor, 1979). Further, if proteolysis of the protein does occur in vitro, one should observe at least a minor polypeptide corresponding to the uncleaved product, as observed with terminal deoxynucleotidyl transferase (Peterson et al., 1984). A premature termination of the nascent peptide chain is also not a unique property of the rabbit reticulocyte lysate system.

The smaller size of the in vitro product could not be due to the synthesis of a protein other than poly(A) polymerase for two reasons. First, the radioactivity in the band corresponding to a 35-kDa protein can be selectively displaced by unlabeled 48-kDa hepatoma poly(A) polymerase. Second, immunoblot analysis using either purified (Stetler & Jacob, 1984) or partially purified preparations (Figure 2) has shown that the molecular weight of the antigen is identical with that of the authentic protein (48 000) which indicates the specificity of the antibodies against the tumor enzyme. If a major immunoreactive contaminant was present in these preparations, other unique bands would have been visible on the autoradiograms obtained from the immunoblot analysis. It is plausible that a particular sequence in the poly(A) polymerase mRNA might prevent complete elongation of the peptide chain preferentially in vitro, probably due to the relative lack of binding of the elongation factors to such a sequence in this system. Translation with RNA denatured by heat or methyl mercuric hydroxide did not alter the product precipitated with immune IgG (data not shown) which rules out the possibility that certain secondary structures capable of causing constraints in the RNA might block translation in vitro.

It seems likely that the smaller size of the product synthesized in the cell-free system is due to lack of glycosylation of poly(A) polymerase in this system. Purified poly(A) polymerase has now been shown to contain approximately 27 glucosamine residues per mole of the enzyme (D. A. Stetler, V. P. Bhavanadan, and S. T. Jacob, unpublished results). It is known that some glycoproteins such as rat α_1-acid glycoprotein synthesized in cell-free systems are as much as 50% smaller than those produced in vivo (Ricca et al., 1981). However, the possibility of other posttranslational modifications such as ADP-ribosylation that is known to alter the electrophoretic mobility of proteins (Ogatha et al., 1981) cannot be ruled out.

Acknowledgments

We thank Dr. Dean A. Stetler for providing poly(A) polymerase antiserum and purified poly(A) polymerase.

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

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