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SYNTHESIS OF DNA POLYMERASE BY IN VITRO TRANSLATION OF CALF RNA

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SUMMARY: Synthesis of α-polymerase in translation mixtures containing calf thymus poly(A^+) RNA was examined by activity gel analysis and by immunobinding with a monoclonal antibody to calf thymus α-polymerase. Activity gel analysis indicated that a DNA polymerase catalytic polypeptide of M_r = ~120,000 had been synthesized. Immunobinding experiments indicated that an immunoreactive polypeptide of about the same size had been formed in vitro. Sucrose gradient centrifugation of calf thymus total RNA revealed that mRNA encoding the ~120,000-M_r DNA polymerase polypeptide sedimented at about 16S. This ~120,000-M_r catalytic polypeptide corresponds in size to an α-polymerase catalytic polypeptide found earlier in crude extracts of calf cells.

A variety of interesting problems can be approached through use of DNA probes for α-polymerase genes. We are attempting to isolate such probes and to examine their specificity by hybrid selection of mRNA capable of encoding α-polymerase in in vitro translation systems. Here we report that translation of calf thymus poly(A^+) RNA resulted in synthesis of at least one polypeptide with DNA polymerase activity. This polypeptide produced a clear signal in the activity gel assay, and a polypeptide of the same size was immunobound by an α-polymerase monoclonal antibody. The M_r of this polypeptide, 110,000 to 120,000, is the same as one of the α-polymerase catalytic polypeptides in crude extracts from calf cells and in some preparations of purified calf α-polymerase (1-4). This demonstration of in vitro synthesis of catalytically active DNA polymerase indicates an approach toward unequivocal identification of α-polymerase cDNA probes using the technique of mRNA hybrid selection.

MATERIALS AND METHODS

Thymus glands from 14-16 week old calves were from Henry W. Stapf Co., Inc., Baltimore, MD. Properties of a rat IgM monoclonal antibody against calf DNA polymerase α (termed MC pol 2) have been described (4). Sodium dodecyl

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sulfate (SDS) was from Bio-Rad. Calf thymus DNA from P.L. Biochemicals was activated by DNase I treatment (5). [α-^{32}P] dTTP and [^{35}S]methionine were from Amersham. Ultrapure guanidine hydrochloride and formalin-fixed Staphylococcus aureus cells (6) were from Bethesda Research Laboratories. The cells were processed before use as recommended by the supplier.

Preparation of RNA from Calf Thymus. Thymus gland was frozen in liquid N_2 immediately after excision and stored at -80°C. The frozen tissue (20 g) was pulverized and then homogenized in 10 volumes 7 M guanidine·HCl, 25 mM sodium acetate, pH 5, 0.1 M 2-mercaptoethanol. Homogenization was at 4°C for 5 min with a Polytron homogenizer and then for 3 min with a Waring blender. An equal volume of $CHCl_3$ was added, and the homogenate was mixed for 3 min in a Waring blender. The resulting mixture was centrifuged and the aqueous phase was obtained. This $CHCl_3$ treatment was repeated four times. RNA then was obtained from the aqueous phase by ethanol precipitation and processed further according to the method described by Paterson and Roberts (7). RNA then was extracted with two volumes of a mixture of phenol:$CHCl_3$:isoamyl alcohol (24:24:1, v/v)(8) and precipitated from the aqueous phase with 0.2 M sodium acetate and two volumes of ethanol. The precipitated RNA was washed with 70% ethanol twice, lyophilized, dissolved in H_2O and stored at -196°C. Poly(A^+)RNA was obtained after two cycles of oligo(dT)-cellulose column chromatography as described by Aviv and Leder (9).

In Vitro Cell-free Synthesis. Both total RNA and poly(A^+) RNA were translated in a BRL reticulocyte lysate system. Incubation was at 30°C for 2 h. The translation mixtures were supplemented with calf liver tRNA at a concentration of 1.8 µg/30 µl. Some reactions were in the presence of 50 µCi [^{35}S]methionine (~ 800 Ci/mmol) per 30 µl translation mixture. At the end of the incubation, aliquots were taken and [^{35}S]methionine incorporation was determined by hot TCA precipitation. The ^{35}S-labeled products were analyzed by electrophoresis in SDS-polyacrylamide gels (10%) as described by Laemmli (10). The gels were fixed in 10% methanol, 5% acetic acid for 30 min, washed with H_2O for 15 min and treated with Autofluor for 2 h. The gels were dried and autoradiography was done at -80°C using Kodak XAR-5 film with a Dupont Lightning Plus intensifying screen.

Assay for DNA Polymerase Activity in Translation Mixtures by the Activity Gel Analysis. Aliquots of the translation mixture were analyzed by electrophoresis on 10% SDS-polyacrylamide gels containing activated DNA (11). The gels were washed and then transferred to a flask containing 40 ml of DNA polymerase assay buffer plus 12.5 µM each of dATP, dCTP and dGTP and 1 µM [α^{32}P]dTTP (~250 µCi). Incubation was at 37°C for 24 h. The gels were washed with two changes of 5% TCA, 1% pyrophosphate for 1 h at 25°C and then with several changes of the same solution at 4°C over a period of 40 h. The gels were dried, and autoradiography was for 24 h at -80°C with Kodak XAR-5 film with a Dupont Lightning Plus intensifying screen (11).

Immunobinding with Monoclonal Antibody to DNA Polymerase α. An aliquot (20 µl) of the translation mixture was diluted four-fold and adjusted to give a solution containing 10 mM methionine in immunobuffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1% aprotinin and 1 mg/ml BSA). The solution was centrifuged for 30 min at 11,000 rpm in a Sorvall HB-4 rotor. The supernatant fraction was then incubated at 4°C for 16 h in a solution containing 100µg/ml monoclonal antibody (MC pol 2) in immunobuffer. After incubation the mixture was added to packed S. aureus cells that were precoated with rabbit anti-rat IgM. Precoating was with 50 µl of a 10% cell suspension in immunobuffer containing 420 µg/ml rabbit anti-rat IgM at 4°C for 1 h with gentle shaking. The mixture was centrifuged at 3,000 rpm for 10 min, and the

cells were washed once with 750 µl of immunobuffer. Reaction between the precoated cells and IgM-translation product complex was at 4°C for 16 h. Cells were collected by centrifugation at 3,000 rpm at 4°C for 10 min. The supernatant fraction was discarded, and the cells were washed, twice with 500 µl of immunobuffer containing 2.5 M KCl and once with 500 µl of 10 mM Tris-HCl, pH 7.3. The cells were collected and the immune complexes dissociated by incubation for 5 min at 25°C in 50 mM Tris-HCl, pH 7.0, 2% SDS, 6 M urea, 5% β-mercaptoethanol (12). The mixture was centrifuged in an Eppendorf microfuge for 1.5 min, and the supernatant fraction was analyzed on a 10% SDS-polyacrylamide gel. Fluorography was conducted as described above. An identical immunobinding experiment was conducted using a nonimmune monoclonal IgM antibody as a control (4).

Sucrose Density Gradient Centrifugation of Total RNA. Total RNA was fractionated on a 5-20% linear sucrose density gradient containing 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Approximately 1.8 mg of total RNA was applied to the gradient in a nitrocellulose tube. Centrifugation was in a Beckman SW 27 rotor at 25,000 rpm at 4°C for 20 h. Fractions of 1 ml were collected from the bottom of the tube. Pools of five fractions each were made, and RNA was recovered by ethanol precipitation.

RESULTS

Typical poly(A^+) RNA preparations from calf thymus were examined by electrophoresis in 1.2% agarose-methyl mercury gels (13). We found that most of the RNA molecules were larger than 28S, suggesting the presence of hnRNA. It was evident also that 18S rRNA had not been completely removed after two cycles of oligo(dT)-cellulose chromatography (not shown). Poly(A^+) RNA was translated *in vitro* using a rabbit reticulocyte lysate system, and to determine whether DNA polymerases had been synthesized, portions of these translation mixtures were subjected to activity gel analysis (1) as described by Karawya et al (11) (Fig. 1). A signal due to an endogenous DNA polymerase catalytic polypeptide was observed at 40,000 M_r (Fig. 1, Lane 1). This band probably was due to the β-polymerase catalytic polypeptide (11, 14). The translation mixture containing poly(A^+) RNA produced an activity band at ~120,000-M_r (Fig. 1, Lanes 2 and 3), and this signal was stronger with the translation mixture containing a higher amount of poly(A^+) RNA (Fig. 1, lane 3). These results suggested that sufficient DNA polymerase accumulated during the incubation to enable detection by activity gel analysis and that α-polymerase messenger RNA was in the poly(A^+) fraction. Results similar to those shown in Figure 1, lane 3, also were obtained with translation mixtures incubated with 7 µg calf thymus total RNA (not shown).

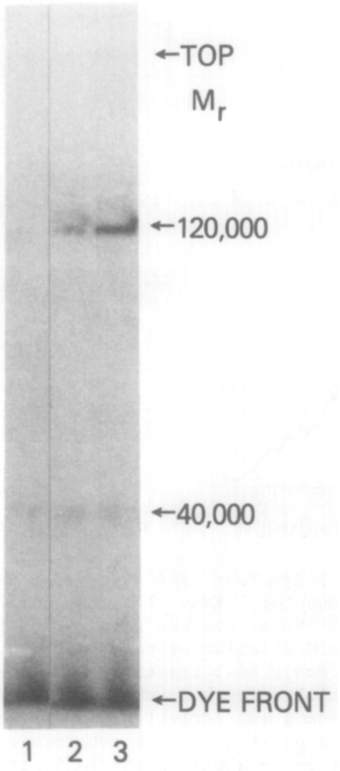

Fig. 1 Autoradiogram showing results of activity gel analysis of in vitro translation products in the reticulocyte lysate system. Translation was conducted with or without exogenous calf thymus poly (A^+) RNA; 80 µM unlabeled L-methionine was present. Aliquots of the translation mixtures, 10 µl, were analyzed using a 10% activity gel. Each sample was mixed with a heterogeneous protein mixture (11). Lane 1, control incubation, no exogenous mRNA. Lane 2, incubation in the presence of 0.9 µg poly(A^+) RNA. Lane 3, incubation in the presence of 2.7 µg poly(A^+) RNA.

Analysis of translation mixtures incubated with rabbit globin mRNA did not reveal a band in the ~ 120,000-M_r region of the gel (not shown), indicating that some type of polynucleotide activation of an endogenous DNA polymerase probably was not responsible for the 120,000-M_r band observed in the translation mixtures containing calf poly(A^+) RNA. In our experience, variation was observed in the intensity of the 120,000-M_r activity band designated in Figure 1. Signal intensity ranged from essentially identical to control incubations (without RNA) to much stronger than that shown in Figure 1. Although reasons for this variation were not fully understood, differences among RNA batches and translation lysates clearly contributed.

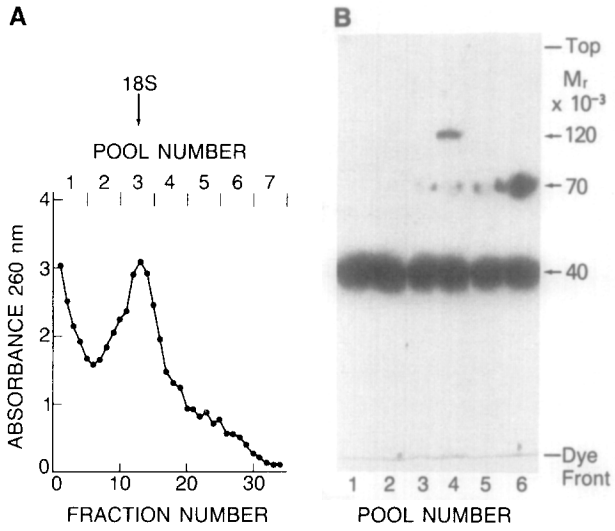

Fig. 2 Enrichment of DNA polymerase mRNA by sucrose density gradient centrifugation of calf thymus total RNA. Panel A, calf thymus total RNA was fractionated in a 5-20% linear sucrose gradient. Absorbence at 260 nm was measured after a ten-fold dilution of each fraction. The top of the gradient is at the right. Pools of 5 fractions each are indicated. Panel B, the RNA pools were translated in the presence of 85 μM L-methionine and the products were analyzed on a 10% activity gel. Aliquots (25 μl) of the translation mixtures were mixed with 9 μl of a heterogeneous protein mixture (11). The autoradiogram of ^{32}P-labeled products is presented. Lanes 1, 2, 3, 4, 5, and 6; translation reactions in the presence of RNA pools 1, 2, 3, 4, 5, and 6, respectively. Each 30 μl translation mixture contained ~ 16 μg of RNA. The activity bands are denoted with arrows and correspond to M_r = ~115,000, 68,000, and 40,000. With unfractionated total RNA the signals obtained in the 110,000-120,000-M_r and ~ 68,000-M_r regions were approximately 5% and 0.5%, respectively, of the corresponding bands shown in lanes 4 and 6 (not shown). A control incubation, without exogenous RNA, was identical to Lane 1.

Strong activity gel signals also could be obtained after fractionating calf thymus total RNA by sucrose gradient centrifugation (Fig. 2). Pooled RNA fractions were translated *in vitro*, and the products were examined by activity gel analysis. With all the translation mixtures, endogenous DNA polymerase activity was observed at 40,000-M_r (Fig. 2A). The results indicated that separation had been achieved of mRNAs encoding the ~120,000-M_r polypeptide and a 68,000-M_r catalytic polypeptide that was not detected with the poly(A^+) RNA fraction; RNA molecules in the pool producing the ~120,000-M_r catalytic polypeptide corresponded to approximately 16S.

In vitro synthesis of α-polymerase polypeptides was evaluated immunologically using a rat IgM monoclonal antibody to calf thymus α-polymerase (4). A rabbit reticulocyte translation mixture with calf thymus total RNA

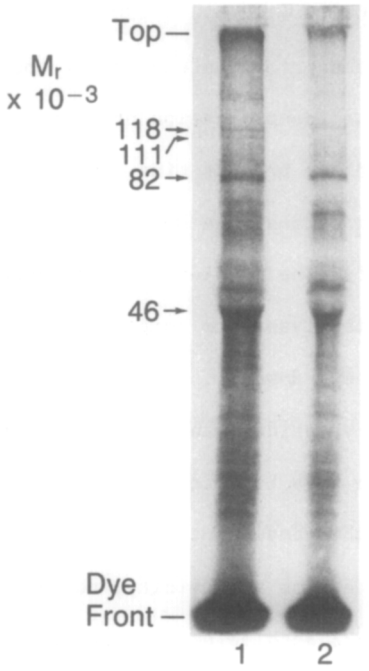

Fig. 3 Autoradiogram showing specific immunobinding of ^{35}S-labeled in vitro translation products using α-polymerase monoclonal antibody. Lane 1, a 20 µl aliquot of a translation mixture containing 40 µg total RNA per 60 µl translation mixture was reacted with 20 µl of 2 mg/ml monoclonal rat IgM antibody (monoclonal polymerase 2 (4)). Lane 2, same conditions as in Lane 1, except that nonimmune rat IgM was used in the immunobinding. Translation reactions were conducted in the presence of 100 µCi of ^{35} S-methionine per 60 µl total translation mixture. The immunobound products were analyzed in a 10% SDS-polyacrylamide gel. M_r values corresponding to four of the radioactive bands are noted.

was probed with this antibody and with a nonimmune rat IgM. Specific immunobinding of ^{35}S-labeled polypeptides was observed, as shown in Figure 3; the most prominent immunobound polypeptide corresponded to M_r = ~118,000.

DISCUSSION

The finding that in vitro synthesis of a putative α-polymerase can be followed by activity gel analysis is important because unequivocal demonstration of in vitro synthesis of this enzyme cannot be based solely upon the widely used approach of immunoprecipitation with a specific antibody. This follows because there is no discrete generally recognized subunit structure of mammalian α-polymerase (for example, reports on calf α-polymerase have identified various enzyme polypeptides with M_r's

of ~ 200,000 (3,4), ~ 150,000 (15,16), ~ 120,000 (1-4,10,14) and 50-76,000 (2-4,14,16)). Further, the primary structure of the enzyme is not yet available, thus ruling out identification of in vitro translation products on this basis. Hence, at the present time, the most reliable method for identification of in vitro translated DNA polymerase α depends upon the catalytic activity itself. The routine solution assay of α-polymerase activity in the translation lysate is complicated by the presence of endogenous DNA polymerases. However, by activity gel analysis, newly formed DNA polymerase polypeptides can be distinguished from endogenous enzymes by the appearance of a band at a M_r different from that of endogenous peptides and the mRNA-dependent enhancement of a specific band. In addition to the advantage of specificity, the activity gel assay may be more sensitive than [^{35}S]methionine incorporation combined with specific immunoprecipitation. This is reasonable because the number of ^{32}P-dNMP residues incorporated per enzyme molecule may be much higher (11) than the number of methionine residues per enzyme molecule. It must be recognized, however, that the activity gel assay, cannot detect DNA polymerase subunits that are inactive as individual proteins. Also, the efficiency of α-polymerase detection in the activity gel assay appears to be low (11), and some enzyme preparations with high activity in routine solution assays are not active in the gel assay (14). In spite of these limitations, the present study demonstrates that activity gel analysis can be used for identification of mRNA encoding the ~120,000-M_r mammalian catalytic polypeptide, and immunobinding results with an α-polymerase monoclonal antibody provided supporting evidence that an α-polymerase polypeptide of this size had been formed in vitro. Thus, the identification of a DNA probe for this enzyme is possible through hybrid selection of mRNA followed by in vitro translation.

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