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Mammalian α-Polymerase: Cloning of Partial Complementary DNA and Immunobinding of Catalytic Subunit in Crude Homogenate Protein Blots†

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ABSTRACT: A new polyclonal antibody against the α-polymerase catalytic polypeptide was prepared by using homogeneous HeLa cell α-polymerase. The antibody neutralized α-polymerase activity and was strong and specific for the α-polymerase catalytic polypeptide (M_r 183 000) in Western blot analysis of crude extracts of HeLa cells. The antibody was used to screen a cDNA library of newborn rat brain poly(A+) RNA in λgt11. A positive phage was identified and plaque purified. This phage, designated λpolα1.2, also was found to be positive with an antibody against *Drosophila* α-polymerase. The insert in λpolα1.2 (1183 base pairs) contained a poly(A) sequence at the 3′ terminus and a short in-phase open reading frame at the 5′ terminus. A synthetic oligopeptide (eight amino acids) corresponding to the open reading frame was used to raise antiserum in rabbits. Antibody affinity purified from this serum was found to be immunoreactive against purified α-polymerase by enzyme-linked immunosorbent assay and was capable of immunoprecipitating α-polymerase. This indicated the λpolα1.2 insert encoded an α-polymerase epitope and suggested that the cDNA corresponded to an α-polymerase mRNA. This was confirmed in hybrid selection experiments using pUC9 containing the cDNA insert and poly(A+) RNA from newborn rat brain; the insert hybridized to mRNA capable of encoding α-polymerase catalytic polypeptides. Northern blot analysis of rat brain poly(A+) RNA revealed that this mRNA is ~5.4 kilobases.

Evidence from inhibitor studies points to a synthetic role of α-polymerase in genomic DNA replication in mammalian cells (Fry, 1982). Activity of α-polymerase is induced as quiescent cells are stimulated to become mitotically active (Fry, 1982; Bollum, 1975; Chang & Bollum, 1973), and conversely, levels of the enzyme decline as actively growing cells become mitotically quiescent (Chang et al., 1973). The mechanisms by which α-polymerase levels are regulated are not understood and are a subject of research interest in these and several other laboratories. Our current approach to this problem is to develop molecular probes for the α-polymerase mRNA and gene. Another important intermediate step has been the definition of α-polymerase catalytic subunits in their native undegraded form in a growing mammalian cell. Karawya et al. (1984) used monoclonal antibody immunoprecipitation and subsequent enzymatic activity analysis to identify a 180-190-kDa[1] protein as the predominant α-polymerase catalytic subunit in cultured monkey cells. Similar results were obtained by Masaki et al. (1984) with calf thymus. For several reasons, we consider immunoblotting analysis of a crude cell extract a much more reliable method than immunoprecipitation for identification of a native polypeptide. Hence, attempts to confirm the immunoprecipitation results were conducted by probing protein blots with monoclonal antibodies to α-polymerases of calf thymus (Swack et al., 1985) or human KB cell (Tanaka et al., 1982). These experiments were not successful. Therefore, in the present work we developed a new specific polyclonal antibody to α-polymerase that gives strong signals

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[1] Abbreviations: kDa, kilodalton(s); SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; kb, kilobase(s); MOPS, 3-(*N*-morpholino)propanesulfonic acid; PBS, phosphate-buffered saline; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SSC, standard saline citrate; BSA, bovine serum albumin; CSA, chicken serum albumin; ADH, adipic dihydrazide; ELISA, enzyme-linked immunosorbent assay; bp, base pair(s).

in protein blot analysis. This antibody, which was raised against a homogeneous preparation of the HeLa α-polymerase catalytic polypeptide (M_r 183 000), neutralized α-polymerase activity. The antibody reacted specifically with a polypeptide of M_r 183 000 when tested against protein blots of the crude extract of HeLa cells. Additional experiments described in this paper apply the new antibody to isolation of rat cDNAs that have properties consistent with those expected of copies of α-polymerase mRNAs.

Materials and Methods

Production of Antibody against HeLa DNA Polymerase α Core. Homogeneous HeLa DNA polymerase α (Vishwanatha et al., 1986) was electrophoresed in a preparative SDS–polyacrylamide gel (Chen et al., 1979). Polypeptides were stained with Coomassie Blue, and a single band of M_r 183 000 was visible (Figure 1A). This polypeptide was excised from the stained gel, and a rabbit was injected subcutaneously with 100 μg of the polypeptide homogenized in complete Freund's adjuvant. A second injection of 100 μg in incomplete Freund's adjuvant was administered 3 weeks after the first injection, and a third injection was administered 4 weeks after the second injection. The rabbit was bled 1 month after the last injection. Typical radioimmunoassay (Swack et al., 1985) titration curves of antiserum against the HeLa α-polymerase 183-kDa polypeptide revealed that immunobinding was dependent upon enzyme amount and antiserum dilution; with microtiter dish wells exposed to 200 ng of enzyme, the titer was 1:200. The IgG fraction of the antiserum was purified as described (Swack et al., 1985).

Electrotransfer to Nitrocellulose and Immunobinding, i.e., the "Western Blot" Procedure. After electrophoresis in a 7.5% SDS–polyacrylamide gel, polypeptides in the gel were electrotransferred to nitrocellulose paper (0.20-μm pore size) according to the method described by Towbin et al. [1979; see also Young et al. (1984)]. The marker proteins and respective kilodalton values used were myosin (200), *Escherichia coli* RNA polymerase (165 and 155), β-galactosidase (116), phosphorylase *b* (92.5), serum albumin (66), ovalbumin (45), carbonic anhydrase (31), and soybean trypsin inhibitor (21.5). The nitrocellulose sheets after rehydration were incubated in 10% normal goat serum at 25 °C for 16 h. After the sheets were washed with water, they were incubated with the anti-α-polymerase IgG, and immunobinding was measured with a peroxidase system as described (Swack et al., 1985). In a typical experiment, the protein concentration of the anti-α-polymerase IgG was 40 μg/mL and the second antibody was at 50 μg/mL goat IgG fraction against rabbit IgG.

Preparation of HeLa Cell Extract. Cell pellets were homogenized by sonic disruption in approximately 3 volumes of 20 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 4 mM magnesium acetate, 0.25 M sucrose, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 mM aminoacetonitrile, 0.1 mg/mL egg trypsin inhibitor, and 10 mM sodium bisulfite. The homogenates were centrifuged at 40000*g* for 30 min at 4 °C. The clear supernatant fractions (~1.8 mg/mL protein) were used immediately or kept frozen at −70 °C. For homogenization in 10% trichloroacetic acid, 0.2 g of packed cell pellet was mixed with 5 mL of 10% TCA at 4 °C. The mixture was subjected to sonic disruption and then to centrifugation at 12000*g* for 5 min. The pellet fraction was suspended in 1.5 mL of SDS–buffer solution as described by Young et al. (1984), except that Tris base was used to adjust pH and heating was for 30 s only. The solution was diluted with 1.5 mL of the SDS–buffer solution, and 10–50 μL was taken for gel electrophoresis and Western blot analysis.

Screening of α-Polymerase Recombinant Clones. Rabbit anti-HeLa DNA polymerase α IgG at 33 μg/mL was used for immunological screening of a λgt11 cDNA library prepared from newborn Sprague-Dawley rat brain poly(A+) RNA (Zmudzka et al., 1986). One nanogram of HeLa DNA polymerase α could be readily detected with this IgG in a dot-blot assay (Renart et al., 1979). Approximately 2×10^6 recombinant phage were cultured on a lawn of *E. coli* Y1090 on 150-mm Petri plates for 2 h at 42 °C as described by Young and Davis (1983). Goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) was used for detection of positive phage. The recombinant phage λpolα1.2 and four others were cloned by plaque purification.

Subcloning of α-Polymerase cDNA Fragments. Preparation of recombinant λ DNA was by the plate lysate method described by Maniatis et al. (1982). *Eco*RI digestion of DNA from clones λpolα1.2, 11.1, 8.1, 5.1, and 15.2 suggested that the insert in all cases was 1.2 kb. The detailed restriction map of all five independently isolated clones was identical also. Therefore, clone λpolα1.2, taken as representative, was used for subsequent studies. The 1.2-kb cDNA insert of λpolα1.2 was excised with *Eco*RI, isolated on a low melting point agarose gel, and ligated to *Eco*RI-cut and phosphatase-treated pUC9 (Vieira & Messing, 1982). The ligation mixture was used to transform *E. coli* JM 83 to ampicillin resistance (Amp^R). A plasmid preparation purified on CsCl–ethidium bromide gradients from the Amp^R *E. coli* transformant pUC9-PKα1.2 was used for all further studies.

Northern and Southern Blot Analysis. Poly(A+) RNA of rat brain and rat cells PC-12 were prepared as described (Detera-Wadleigh et al., 1984). Poly(A+) RNA (10 μg) was denatured by heating at 60 °C for 15 min in 2.2 M formaldehyde and 50% (v/v) formamide and subjected to electrophoresis in a 1.5% agarose gel containing 2.2 M formaldehyde, 20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA. Genomic DNA (10 μg) was obtained as described (Blin & Stafford, 1976), cleaved with restriction endonucleases, and then fractionated in a gel of 0.8% agarose. RNA or DNA was electrotransferred to Gene-Screen hybridization transfer membrane and hybridized to the ^{32}P-labeled nick-translated 1.2-kb cDNA insert as described by Zmudzka et al. (1986).

Immunoprecipitation of DNA Polymerase. DNA polymerase α, either in crude extract or as purified enzyme, was immunoprecipitated as an enzyme–IgG complex with protein A–Sepharose beads. The mixture for enzyme–antibody complex formation in a final volume of 20 μL contained 10 μL of either crude extract or purified enzyme dissolved in the homogenization buffer and 10 μL of 50 μg/mL IgG in PBS. This mixture was incubated at 4 °C for 1 h. Then, 30 μL of a mixture of 210 mg/mL protein A–Sepharose in PBS was added, and incubation at 4 °C was continued for 30 min. The mixture was centrifuged in a microfuge, and 5-μL portions of the supernatant fraction were removed for DNA polymerase assay. DNA polymerase activity was measured as described (Karawya, 1984). Each reaction mixture in a final volume of 10 μL contained 50 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 6 mM magnesium acetate, 400 μg/mL bovine serum albumin, 15% glycerol, 180 μg/mL activated calf thymus DNA, and 100 μM dNTP, one ^3H- or ^{32}P-labeled (specific activity 18 000 dpm/pmol). Incubation was at 37 °C for 10 min.

Hybrid Selection and in Vitro Translation. Twenty micrograms of pUC9 DNA containing the λpolα1.2 cDNA insert was linearized with *Eco*RI, denatured by heating, and attached

to DBM paper (Schleicher & Schuell). Hybridization was with 10 μg of newborn rat brain poly(A+) RNA in 10 mM PIPES buffer, pH 6.4, 65% formamide, and 400 mM NaCl at 50 °C for 24 h. The paper was washed at 55 °C in 1 × SSC and 0.5% SDS, and then at 25 °C with 2 mM EDTA, pH 8. Hybridized RNA was removed by 5-in incubation at 65 °C in 10 mM Tris-HCl, pH 7.5, and 99% formamide. RNA was recovered by ethanol precipitation, dissolved in 10 μL of water, heated at 65 °C for 6 min, and placed on ice. Twenty microliters of a translation mixture containing 10 μL of a reticulocyte lysate (Promega Biotec) was added, and the mixture was incubated at 30 °C for 2 h. The entire mixture then was submitted to SDS–polyacrylamide gel electrophoresis (Karawya et al., 1984). Portions of the gel containing polypeptides of interest were cut, and proteins were electroeluted, renatured, and assayed for DNA polymerase activity as described (Karawya et al., 1984).

Preparation of Synthetic Oligopeptide and Rabbit Antiserum Production. The peptide Gly-Pro-Val-Arg-Tyr-Ser-Cys-Arg was synthesized by using an Applied Biosystems, Inc., Model 430A peptide synthesizer. Deprotection and release of the peptide from the PAM resin was accomplished by using anhydrous HF with 10% thioanisole at −5 °C for 2 h. Following ethyl acetate extraction, pure peptide was obtained in a yield of 68%. The peptide was derivatized by reacting 100 mg of peptide with a 1.1 molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, Aldrich) in 5 mL of 0.1 M sodium bicarbonate for 15 min at 25 °C. The derivatized peptide was separated from other reaction products by chromatography on Sephadex G-10 in 0.1 M ammonium bicarbonate and then lyophilized.

Prior to conjugation of the peptide to bovine serum albumin (BSA) or chicken serum albumin (CSA), these proteins first were derivatized with the nucleophilic spacer adipic dihydrazide (ADH) as described by Schneerson et al. (1980). For conjugation, the synthetic peptide (100 mg) was incubated with an equimolar amount of bromoacetyl bromide in 5 mL of 0.1 M sodium bicarbonate for 15 min at 25 °C. To this was added 30 mg of BSA–ADH or CSA–ADH in 0.1 M sodium bicarbonate, and the reaction was continued for 18 h at 25 °C. The conjugates were purified by using a Sephadex G-50 column, lyophilized, and dissolved in PBS at 2 mg/mL. A 0.5-mL volume of the BSA–ADH–peptide solution was mixed with an equal volume of complete Freund's adjuvant. This mixture was injected subcutaneously at multiple sites in a New Zealand White rabbit. Three weeks later 0.5 mL of CSA–ADH–peptide was mixed with an equal volume of incomplete Freund's adjuvant and injected as before. The rabbit was bled after 3 weeks; the serum was positive by ELISA (Voller et al., 1976), using immobilized BSA–ADH–peptide. The synthetic peptide was conjugated to Sepharose by using activated Sepharose CH-4B as recommended by the manufacturer (Pharmacia Fine Chemicals), and the antiserum (10 mL) then was stirred overnight with 9 mL of the Sepharose/peptide. The serum was separated from the Sepharose on a column, and the column was washed with PBS, followed by 25 mL of 2 M sodium chloride. The antibody against the peptide was eluted with 4 M magnesium chloride, and the eluate then was dialyzed against PBS. IgG concentration and purity were evaluated by SDS–polyacrylamide gel electrophoresis. This IgG was >90% pure, as judged by the absence of other stained protein material.

Results

Antibody to α-Polymerase and Western Blot Probing. The HeLa α-polymerase (M_r 183 000) catalytic subunit was purified to homogeneity (Figure 1A) and then used to raise antiserum in a rabbit. Samples of serum were obtained periodically and examined for α-polymerase antibody by solid-phase radioimmunoassay with immobilized, purified HeLa α-polymerase (Swack et al., 1985). High-titer antiserum was obtained eventually, and the IgG fraction was prepared. This antibody was capable of immunobinding to purified α-polymerase in Western blotting analysis (Figure 1B) and neutralization of HeLa α-polymerase activity but not HeLa β-po-

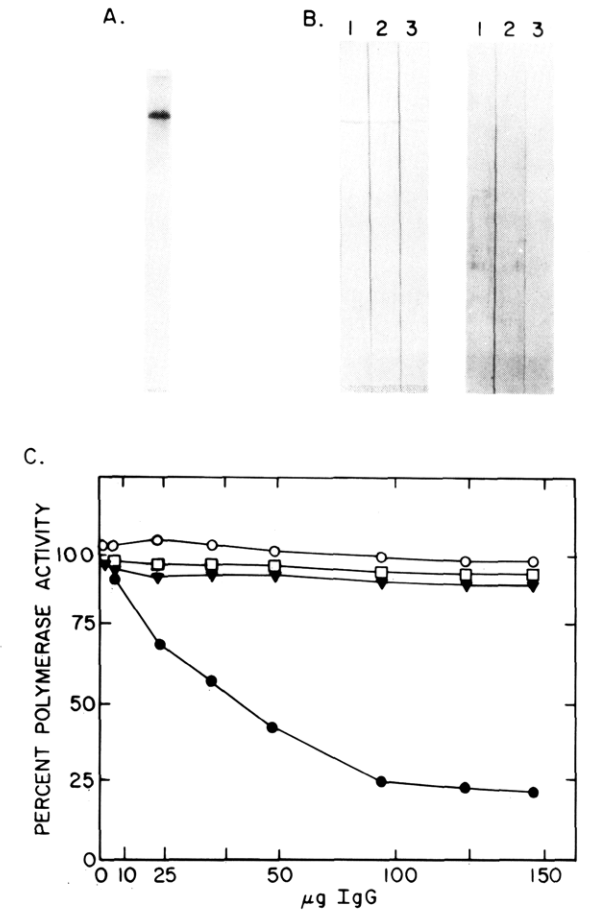

FIGURE 1: Specificity of anti-HeLa cell α-polymerase antiserum for DNA polymerase α catalytic subunit. (A) Purified DNA polymerase α catalytic core was subjected to SDS–polyacrylamide gel electrophoresis as described previously (Vishwanatha et al., 1986). The only Coomassie Blue stained material seen formed a band corresponding to M_r 183 000. This band was excised and used as the immunogen for production of rabbit anti-α-polymerase IgG by the procedure described under Materials and Methods. (B) Immunoblot analysis of HeLa α-polymerase using purified anti-α-polymerase IgG and control (preimmune) rabbit IgG. HeLa α-polymerase catalytic core (α_3) (Vishwanatha et al., 1986), 0.8 μg mm^2, was electrophoresed in a 7.5% SDS–polyacrylamide gel, electrotransferred to nitrocellulose paper, and developed with anti-α-polymerase IgG or preimmune IgG as described under Materials and Methods. Left panel, lanes 1–3: Anti-α-polymerase IgG at concentrations of 45, 18, and 9 μg/mL, respectively. Right panel, lanes 1–3: Preimmune IgG at concentrations of 108, 43, and 21 μg/mL, respectively. (C) Enzyme neutralization assays with anti-α-polymerase IgG. Aliquots of the purified HeLa cell DNA polymerase α catalytic subunit (0.5 unit) (●, ○), HeLa cell DNA polymerase β (0.2 unit) (□), and HeLa cell DNA polymerase γ (▼) were preincubated with anti-HeLa cell polymerase α IgG (0.6 mg/mL) or preimmune rabbit IgG (○) (0.6 mg/mL) in the presence of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 5 mg/mL bovine serum albumin at 4 °C for 1 h. Following the preincubation, the samples were assayed for DNA polymerase α activity as described under Materials and Methods and for polymerase β and γ activities as described previously (Chu & Baril, 1975). The 100% values represent, for DNA polymerase α, 560 pmol of dNMP incorporated; for polymerase β, 320 pmol of dNMP incorporated; and for polymerase γ, 130 pmol of dNMP incorporated.

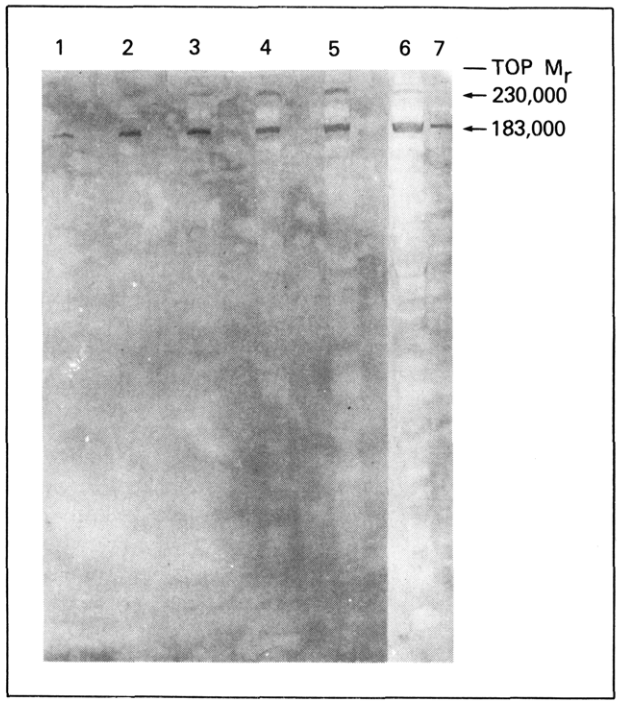

FIGURE 2: Antibody probing of protein blot of initial crude extract of growing HeLa cells. Experiments were conducted with anti-α-polymerase IgG at 40 μg/mL as described under Materials and Methods. Different amounts of crude extract from cells cultured under two types of conditions were denatured and applied directly to a 7.5% SDS–polyacrylamide slab gel. Proteins were resolved by electrophoresis and eventually transferred to a nitrocellulose sheet. The two types of culture conditions were cells maintained in logarithmic growth phase and cells in active growth after release from hydroxyurea block. Increasing amounts of protein from the growing cell extract were added to lanes 1–5 as follows: 6, 15, 30, 45, and 60 μg. Lanes 6 and 7 contained 60 and 6 μg, respectively, of extract from the block-released cells. Probing with IgG from the preimmune serum (40 μg/mL) failed to reveal any significant signals (not shown).

lymerase or γ-polymerase activities (Figure 1C).

The antibody was used in Western blot analysis of the initial crude extract of HeLa cells. Homogenates of synchronously growing (Chu & Baril, 1975) or log-phase cells were prepared in a buffer solution containing four protease inhibitors, and the crude extracts were denatured and electrophoresed. The antibody then was used to probe Western blots (Figure 2). With both extracts a strong signal was observed in only one region of the gel, corresponding to M_r 183000. At higher levels of crude extract, a second signal was present, corresponding to a M_r ~230000 polypeptide. This signal appeared to be about 20-fold less abundant than the signal at M_r ~183000.

The results in Figure 2 demonstrate both the specificity of the antibody and its utility in probing protein blots. In addition, the results confirm that the immunogen, the M_r 183000 polypeptide (Vishwanatha et al., 1986), is a native species of α-polymerase in growing HeLa cells rather than a degradation product of proteolysis during purification. This possibility was evaluated further by homogenization of log-phase HeLa cells directly in 10% trichloroacetic acid (Young et al., 1984). Such homogenates should accurately reveal the in vivo state of the protein since direct homogenization in acid should block proteolytic activity. Analysis of the crude extract prepared in this manner gave results very similar to those in Figure 2 (not shown).

Isolation of a λgt11 Clone and Sequencing of the cDNA Insert. We found that the antibody cross-reacted with purified α-polymerase from mouse myeloma and with partially purified α-polymerase from rat cells. Therefore, we assumed the antibody could be used to select α-polymerase clones from a cDNA expression library of rodent poly(A+) RNA (Cobianchi et al., 1986; Zmudzka et al., 1986). Screening of a newborn rat brain cDNA library in λgt11 was conducted by using a total of 3×10^7 recombinant phage distributed in 15 150-mm Petri dishes. Thirty-two positive phage were identified, and five of them eventually were cloned by plaque purification with six cycles of rescreening. One of the clones, designated λpolα1.2, was selected for detailed study. Some immunological properties of the plaques produced by the purified clone are illustrated in Figure 3. The signal produced with the antibody was much stronger than that obtained with unselected library phage, as revealed by an experiment in which λpolα1.2 was mixed with the initial library and then the mixture was probed with the antibody. The phage did not give a signal when probed with IgG from the preimmune serum.

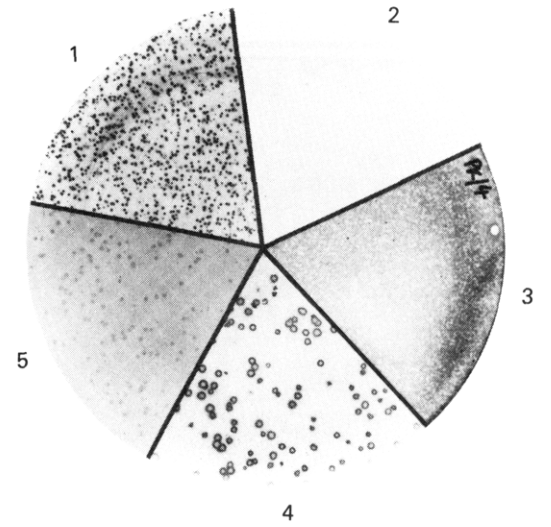

FIGURE 3: Signal produced by purified λpolα1.2 probed with various rabbit IgGs after staining with a horseradish peroxidase based dye system. Sections of five different blots are shown: section 1, purified λpolα1.2 at 10^4 phage per 100-mm dish, probed with anti-HeLa α-polymerase IgG; section 2, same as above, except probe was the rabbit preimmune serum IgG; section 3, a mixture of 100 λpolα1.2 phase and 2×10^6 library phage per 150-mm dish, probed with anti-HeLa α-polymerase IgG; section 4, purified λpolα1.2 at 500 phage per 100-mm dish, probed with anti-*Drosophila* α-polymerase IgG; section 5, purified λpolα1.2 at 10^3 phage per 100-mm dish, probed with affinity-purified IgG against the eight amino acid oligopeptide described in the text.

λpolα1.2 was evaluated by using several independently derived antibodies to α-polymerase. The plaques failed to react with two monoclonal antibodies (MC pol 1 and 2) to calf thymus α-polymerase (Swack, 1985), with a monoclonal antibody (132-20) to KB cell α-polymerase (Tanaka et al., 1982), or with a polyclonal antibody to β-polymerase (Tanabe et al., 1984). However, a polyclonal antibody specific for the *Drosophila* α-polymerase catalytic polypeptide (Sauer & Lehman, 1982) gave a strong signal (Figure 3, section 4) similar to that observed with the antibody prepared here.

The nucleotide sequencing strategy and partial restriction map for the cDNA insert in λpolα1.2 are shown in Figure 4. The insert contained 1183 bp, including a poly(A) sequence at the 3′ end. The complete nucleotide sequence of the cDNA is shown in Figure 5. The sequence had numerous stop codons throughout in all three reading frames and did not have a long open reading frame; the reading frame that was in phase with the β-galactosidase-coding region of λgt11 extended only 22 nucleotides from the 5′ end of the insert to a termination codon, TAA, at residues 23–25. Hence, the open reading frame of the cDNA responsible for the epitope appeared to encode only

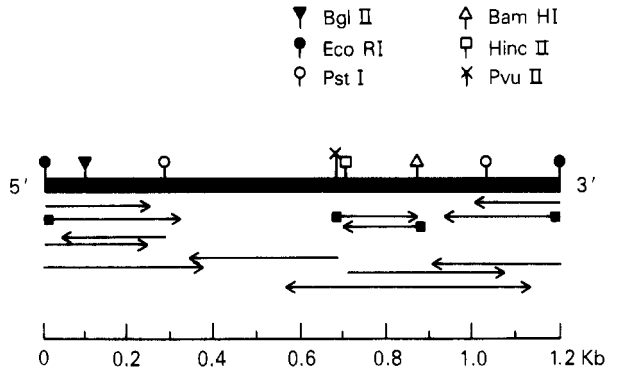

FIGURE 4: Partial restriction map and sequencing strategy for the λpolα1.2 cDNA insert. Sequencing by the method described by Sanger (1980) was in pUC9 or M13 (—); sequencing by the method described by Maxam and Gilbert (1980) was with fragments cut from the cDNA as appropriate (■).

the seven amino acid sequence Pro-Val-Arg-Tyr-Ser-Cys-Arg, presumably corresponding to the C-terminal end of the M_r 183 000 α-polymerase polypeptide. The remainder of the cDNA sequence contained a putative polyadenylation signal 19 residues 5′ of the poly(A) sequence and three T-rich sequences 16 or 17 residues long (Figure 5).

Northern Blot Analysis of Poly(A+) RNA of Newborn Rat Brain. Northern blot analysis of newborn rat brain poly(A+) RNA was conducted with the λpolα1.2 insert as probe. Poly(A+) RNA from a rat cell line, PC-12, in growth phase was included for comparison. One sharp hybridizing band was observed with both RNA samples, corresponding to a transcript of approximately 5400 bases. Assuming that this mRNA contained a 1161-bp 3′ untranslated region as noted in Figure 5, the sequence was long enough to encode a protein of 1412 amino acids or about 180 000 daltons, a value in good agreement with the molecular weight of the α-polymerase catalytic polypeptide (see Figures 1 and 2; Karawya et al., 1984; Vishwanatha et al., 1986).

Verification of the λpolα1.2 Insert. We prepared a synthetic oligopeptide of eight amino acids corresponding to the sequence of the open reading frame at the 5′ end of the cDNA, Gly-Pro-Val-Arg-Tyr-Ser-Cys-Arg. Rabbit antiserum to this synthetic oligopeptide was raised, and the specific IgG fraction was prepared by immunoaffinity chromatography using a column of the oligopeptide coupled to Sepharose. This IgG gave a positive signal when tested against plaques formed by the cloned phage λpolα1.2 (see Figure 3, section 5). The antibody then was tested for immunobinding to homogeneous α-polymerase from HeLa cells by using the ELISA method (Figure 6). Immunobinding was observed and was dependent upon both enzyme amount and antibody dilution. Immunobinding was completely abolished by the cDNA-derived oligopeptide but not by an unrelated oligopeptide. Immunobinding between the antibody and native α-polymerase was examined further by using an immunoprecipitation technique in which enzyme–IgG complexes were collected on protein A–Sepharose beads (Table I). Partially purified or purified α-polymerase from rat or human cells was removed from solution by the immune IgG but not by IgG from the preimmune serum. We conclude from these results that the sequence of the λpolα1.2 cDNA insert specified an amino acid sequence that contained an α-polymerase epitope.

The identity of the λpolα1.2 cDNA insert was further evaluated by hybrid selection and in vitro translation of newborn rat brain poly(A+) RNA (Detera-Wadleigh et al., 1984). After incubation, translation mixtures were electrophoresed in an SDS–polyacrylamide gel; regions of the gel corresponding

Table I: Immunoprecipitation of α-Polymerase Activity by Antibody to cDNA-Derived Oligopeptides

α-polymerase preparation[a]	amt of DNA polymerase in soln (nmol of dNMP/h) with IgG (25 μg/mL)		% immunoprecipitation
	preimmune	immune	
N1S1 crude extract	35	10	72
HeLa crude extract	10	2	80
purified HeLa	3	0.5	83

[a] N1S1 rat cells (Hoffee et al., 1982) and HeLa cells were in growth phase. The extracts were prepared as described under Materials and Methods.

Table II: DNA Polymerase Activity after Hybrid Selection and in Vitro Translation of Newborn Rat Brain Poly(A+) RNA

cDNA insert in immobilized pUC9 (20 μg)	renatured polypeptides	hybrid selection step (pmol of dNMP/h)[a]	
		plus poly(A+) RNA (10 μg)	minus poly(A+) RNA
λpolα1.2	p70, p120, p180	27	4
	p40	2	3
none	p70, p120, p180	5	5
	p40	2	2
λpolβ10	p70, p120, p180	4	5
	p40	15	3

[a] Translation mixtures were electrophoresed in a 10% SDS–polyacrylamide gel. Regions of the gel corresponding to the indicated polypeptides were sliced; proteins were electroeluted, renatured, and assayed for polymerase activity (Karawya et al., 1984). Each sample was assayed separately in triplicate. Average values from two experiments are shown. The rabbit reticulocyte lysate based translation mixture was incubated for 2 h at 30 °C (Detera-Wadleigh et al., 1984). The DNA polymerase incubation was for 30 min at 37 °C with activated DNA as template primer as described (Karawya et al., 1984). The rat β-polymerase cDNA was described by Zmudzka et al. (1986).

to the three known α-polymerase catalytic polypeptides (p70, p120, and p183) were sliced; proteins then were electroeluted, separated from SDS and renatured, and assayed for DNA polymerase activity (Karawya et al., 1984). In the experiment shown in Table II, pUC9 containing a cDNA for rat β-polymerase (Zmudzka et al., 1986) was included for comparison. The β-polymerase cDNA selected mRNA capable of encoding an enzyme polypeptide of about M_r 40 000, which is the size of both native β-polymerase and the mRNA long open reading frame (Zmudzka et al., 1986). The λpolα1.2 cDNA selected mRNA capable of encoding the three known α-polymerase catalytic polypeptides (Spanos et al., 1981; Albert et al., 1982; Karawya & Wilson, 1982; Scovassi et al., 1982) which are about M_r 70 000, 120 000, and 183 000. Approximately equal activity was observed with the renatured samples of each of these polypeptides. The reticulocyte lysate that was not supplemented with poly(A+) RNA contained substantial amounts of both α- and β-polymerase catalytic polypeptides. No increases over the endogenous levels were observed when pUC9 alone was used in the hybrid selection step. These results confirm that the λpolα1.2 insert corresponded to α-polymerase mRNA. The production of the two catalytic polypeptides of lower molecular weight than the 183 000 polypeptide may have been the result of proteolytic degradation during the 2-h incubation of the translation mixture, although this is by no means certain.

DISCUSSION

We prepared a new polyclonal antibody to the mammalian α-polymerase catalytic polypeptide and found that this antibody gives a strong signal when used as probe in protein

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                                                                        68
C CCC GTT AGA TAC AGC TGT CGG TAA gggtgtaccagctcactaagaaacactactgtcaagattttaa
  Pro Val Arg Tyr Ser Cys Arg ***

                                                                       145
agctcagaccaagtgacacgttaataagaaactaagatctgattgaatttttctaacagtccttgcctcgtggacacg

                                                                       222
ctagaaccggagagcctgtaagtgtgacaggatcaaaactggcctggctggagggttaggtggactttgatctgcag

                                                                       299
tgttccttggtcttcgtttggggaatatttcttgggctgtttgctgaagtgccgtagctgatggatggtgaagtaat

                                                                       376
ttgaacgtacgcgattgtaggttttgttatttgttttaagatttaaaggctggttgtagcatttaaaggtaaacttt

                                                                       453
ttcttcctggtttgcgtagtgttctgcgtgtcttctttagttgaaataggttagtggagaagttaagaaagcggtgg

                                                                       530
tgcccgtgttacgctggtggtaaccagggccgcctacaaactctcagttttgttttgttttttataacagtcccac

                                                                       607
tcgtacaataatgagttcactccttggataaggttgttaatgagggaagagcccttcagacctaatcagctcttact

                                                                       784
ggacccagctgtcaacacttgttgcattggggattaagtttccaacccgtgaatcatgggacagactcaaaccgtag

                                                                       761
cactgggcatgtcgcctaacaatataatgtgactcagtattctgtgagacatgggataatattcctgacctacctcc

                                                                       838
acgggtaggtgcgagcatgagcaaatgctctttataaaacatttggggatcgagcaaatgctctttataaaacattt

                                                                       915
ggggatcctcagcagaggagtatttaagtcctgcacatttttatagtaaccgtccaccacggacttgacacgtgcct

                                                                       992
acgtcccagacgacaccagccaacctacaccattcttctcggaggccgaacggacgcactagattaatgtatactca

                                                                      1069
ttgctatggaaacctgcagggactccttttccttcctgggtgggatttttttttccttttattatgtgcaacagtagc

                                                                      1142
tatttgtaatctaataattttcattaataccaactctgaaagtatttctactcatctgaggttgtgtttgttcataa

                                                                      1183
taaaatgaaatggatctgactgcaaaaaaaaaaaaaaaaaa
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FIGURE 5: Nucleotide sequence of the λpolα1.2 cDNA insert and deduced sequence of the seven amino acid peptide corresponding to the reading frame in phase with the β-galactosidase-coding sequence. The coding strand is shown, 5′ to 3′, and the open reading frame is shown in capital letters. A putative mRNA processing signal (AATAAA) is underscored some 19 nucleotides 5′ of the poly(A) sequence. Three 16 or 17 residue long T-rich regions are noted also.

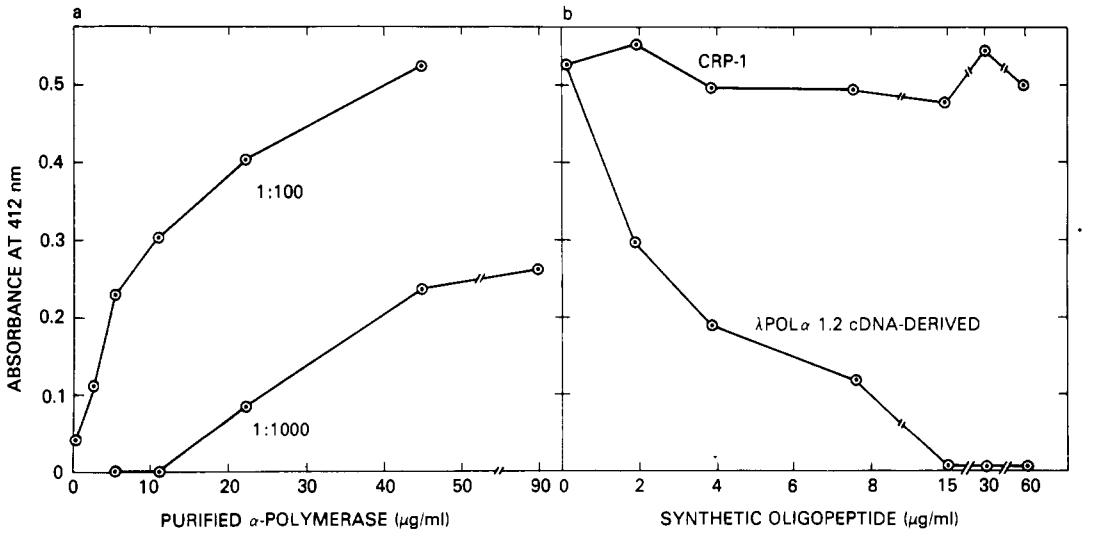

FIGURE 6: Immunobinding in an ELISA test between purified HeLa α-polymerase core enzyme and the immunoaffinity-purified IgG against the eight amino acid oligopeptide described in the text. The IgG solution at 50 μg/mL was diluted as indicated in panel a or was used at a dilution of 1:100 in panel b. In panel b, α-polymerase was at 45 μg/mL for coating wells and the CRP-1 oligopeptide was 12 amino acid residues long and was unrelated to the α-polymerase cDNA sequence.

blotting experiments (Figure 1). The antibody neutralized α-polymerase but not β- or γ-polymerase. We found that the antibody was specific for the M_r 183 000 mammalian α-polymerase catalytic polypeptide (Albert et al., 1982; Faust et al., 1984; Karawya et al., 1984; Vishwanatha et al., 1986) in protein blot analysis of the initial crude extract of HeLa cells (Figure 2). This enabled verification by the Western blot technique that the predominant native form of the α-polymerase catalytic polypeptide in growing primate cells is the M_r 183 000 species. Hence, this unambiguous demonstration of the M_r 183 000 protein in cells is an important step toward understanding the mechanism of α-polymerase. The M_r 183 000 species now has been purified by biochemical and immunological procedures from primate cells and has been renatured from SDS–polyacrylamide gels. Catalytic studies of the protein both alone and in association with accessory proteins will answer questions about the regulation and specificity of α-polymerase during DNA replication. The crude-extract blotting experiments also revealed a minor protein species (M_r 230 000) with an antibody epitope. This species was much less abundant than the M_r 183 000 species, and its precise identity was not determined. This species, however, probably corresponds to an α-polymerase polypeptide of similar size observed by traditional purification (Faust et al., 1984) and monoclonal antibody techniques (Swack et al., 1985; Masaki et al., 1984).

The α-polymerase-specific antibody was used to select a λgt11 recombinant phage (λpolα1.2) capable of expressing an antibody epitope. The cDNA insert in the recombinant phage was 1.2 kb, had a poly(A) sequence at the 3′ end, and hybridized only with a 5.4-kb transcript in the poly(A+) RNA preparation from which the library was derived. This indicated that the cDNA represented only a portion of the 5.4-kb poly(A+) RNA. Sequencing of the cDNA revealed that it contained a poly(A) sequence at the 3′ end and that it did not contain a long open reading frame, suggesting that most of the sequence corresponded to the 3′ untranslated region of the 5.4-kb mRNA. In the extreme 5′ end of the cDNA, the open reading frame that was in phase with the λgt11 β-galactosidase-coding region contained only 22 nucleotides. This sequence specified a seven amino acid oligopeptide, which corresponds to the approximate size of a single antigenic determinant (Atassi, 1975). The oligopeptide sequence contained one proline residue and two arginine residues and was predicted to be antigenic on the basis of an overall hydrophilicity value of 0.5 (Hopp & Woods, 1981). We found that an antibody raised against the synthetic oligopeptide with the cDNA-deduced sequence reacted with purified α-polymerase in ELISA (Figure 6), and this antibody also could be used in immunoprecipitation of α-polymerase activity (Table I). The results indicated that α-polymerase and the cDNA-deduced oligopeptide had a common epitope. As expected, the antibody to the oligopeptide reacted with an epitope in plaques formed by the cloned phage λpolα1.2. The plaques also contained an epitope for an independently derived antibody specific for the α-polymerase catalytic polypeptide, i.e., from *Drosophila melanogaster* (Figure 3). Finally, we found that the cDNA subcloned in pUC9 was capable of hybridizing to α-polymerase mRNA, as revealed by in vitro translation experiments (Table II). These results, taken together, strongly suggest that the cDNA corresponded to be 5.4-kb α-polymerase mRNA.

Our results from Northern blot analysis demonstrate that the 1.2-kb cDNA is a specific probe for the 5.4-kb transcript. Under the stringent RNA–DNA hybridization conditions used, nonspecific binding of the probe was not detected, allowing use of unfractionated total RNA in addition to poly(A+) RNA. The abundance of the 5.4-kb transcript was about 100-fold less than the transcript(s) for rat helix-destabilizing protein (Cobianchi et al., 1986) and was about 4-fold more than the transcript for rat β-polymerase (Zmudzka et al., 1986). In an initial attempt to study the regulation of α-polymerase synthesis, we found that the abundance of the 5.4-kb α-polymerase mRNA in newborn (5–10 day old) and adult rat brain was about the same (not shown). This was surprising as the level of α-polymerase activity in adult rat brain has been reported to be lower than in newborn rat brain (Hubscher et al., 1977). In addition, growing and differentiated (2 weeks of treatment with nerve growth factor) rat PC-12 cells also had the same abundance of the 5.4-kb transcript. These results, although preliminary, suggest that in some cases levels of α-polymerase mRNA and enzymatic activity may not correlate. Further studies on changes in the synthesis and accumulation of α-polymerase mRNA should now be possible by using the 1.2-kb cDNA.

Finally, the 1.2-kb insert was used to probe DNA blots of restriction enzyme treated genomic DNA from rat, mouse, and hamster cells. A relatively simple hybridization pattern was observed with each type of DNA and with each of six restriction enzymes (not shown). The autoradiogram exposure time required to detect the signals (72 h) was much longer than that required for high-copy-number DNA elements in our experience and was about the same as that for single-copy genes. Portions of the 1.2-kb insert should be useful probes for isolation of α-polymerase genomic clones and eventually of cDNAs that are full-length copies of the 5.4-kb mRNA.

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Registry No. α-DNA polymerase, 9012-90-2.

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