Purification of a DNA Polymerase-DNA Primase Complex from Calf Thymus Glands*

Lucy M. S. Chang, Elizabeth Rafter, Christel Augl, and F. J. Bollum

From the Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

An immunoabsorbent column, prepared by covalently linking mouse monoclonal anti-calf thymus DNA polymerase-α to Protein A-Sepharose, was used as the primary purification step for rapid isolation of DNA polymerase-α from calf thymus-gland extracts. In a 4-step procedure consisting of the removal of nucleic acids by protamine sulfate precipitation, chromatography on the immunoabsorbent column, desalting on Sephadex G-50, and removal of bovine immunoglobulins on Protein A-Sepharose, DNA polymerase-α activity was purified about 5000-fold from the crude extract with greater than 40% recovery of total enzyme activity.

The antibody column-purified DNA polymerase-α fraction contains a DNA primase activity that is efficient in replication of single-stranded DNA and poly(dT) when rNTPs are included in the replication reactions. Synthesis by calf thymus DNA polymerase-primase is totally dependent on added template. Complete replication of circular single-stranded phage DNA is achieved with polymerase-primase producing a nicked circular DNA containing oligoribonucleotide primer in the final product. Primers synthesized with single-stranded phage DNA as template were up to 10 nucleotides long when dNTPs were omitted from the reaction and 8 or less nucleotides long when dNTPs were present. Primers synthesized using poly(dT) consisted of three populations when dATP was absent from the reaction, averaging 20 nucleotides, 10 nucleotides, and 3–4 nucleotides. The 20-nucleotide population was not found when dATP was included in the reaction.

Purification of DNA polymerase-α from eukaryotic sources by conventional procedures requires lengthy processing and is often hindered by problems with proteolysis (1–6). With the development of monoclonal antibodies to human DNA polymerase-α (7) and immunoaffinity chromatography, rapid purification schemes have been developed for DNA polymerase-α from human KB cells (8) and from calf thymus glands (9).

The immunoaffinity-purified human KB cell DNA polymerase-α was found to contain a tightly bound DNA primase and is capable of replicating single-stranded circular phage DNA and poly(dT) when rNTPs are supplied in the replication reactions (8, 10). Purification of the calf thymus DNA polymerase-α, utilizing a monoclonal antibody to human KB cell enzyme (9) produced a high-molecular-weight enzyme complex, but the purification scheme used resulted in 1% activity recovery.

In the present work, we describe a procedure for rapid purification of DNA polymerase-α from calf thymus glands on a reusable immunoabsorbent column with excellent recovery of enzyme activity. The purified enzyme contains DNA primase activity that is closely coupled to the deoxynucleotide polymerization process. We believe that analysis of several forms of DNA polymerase-primase complex will be necessary to reveal the components of the eukaryotic replication process.

EXPERIMENTAL PROCEDURES

Materials

Unlabeled rNTPs1 and dNTPs were purchased from Sigma. [8-3H]dATP, [CH3-3H]dGTP, [α-32P]dATP, [γ-32P]dATP, [α-32P]dCTP, and [α-32P]dGTP were from New England Nuclear. Bacteriophage fd DNA was from Miles. Restriction endonuclease HaeIII, Escherichia coli DNA polymerase I, and SV40 DNA were from Bethesda Research Laboratories. φX174 viral DNA and φX174 RF I DNA were prepared as described by Kunkel and Loeb (11). Herpes simplex type I DNA was provided by Dr. William Ruyechan of Uniformed Services University of the Health Sciences. Calf thymus DNA and RNase-free bovine pancreatic DNase I were from Worthington. Poly(dT), chain length of 3000, was synthesized with terminal deoxynucleotidyltransferase (12). Oligodeoxynucleotides were prepared as previously described (13). Protein A-Sepharose was from Pharmacia. Proteins for molecular weight standards were from Bio-Rad. Other chemicals are reagent grade.

Mouse monoclonal antibodies to calf thymus DNA polymerase-α were prepared by a procedure essentially as described for the panel of mouse monoclonal antibodies to human terminal transferase (14). Four different monoclonal antibodies (all are IgG1 subtype) were used for experimental purification of calf thymus DNA polymerase-α.

Mouse monoclonal antibody to Protein A-Sepharose was coupled to Protein A-Sepharose with dimethyl suberimidate by mixing mouse IgG1 (purified from hybridoma culture supernatant on a Protein A-Sepharose column) with Protein A-Sepharose in 0.2 M triethylammonium HCl buffer at pH 8.7 at a ratio of 2 mg of IgG1/ml of Protein A-Sepharose. After mixing for 1 h at room temperature, the suspension was centrifuged in a clinical centrifuge, and the amount of IgG1 was determined by A280. Binding was usually greater than 80%. After resuspension in the same solution, solid dimethyl suberimidate was added with mixing to 3 mg/ml. Mixing was allowed to continue for 4 h at room temperature. The gel suspension was then poured into a column and washed with at least 10 volumes each of phosphate-buffered saline (pH 7.4), 0.2 M NaCl, 3.5 M MgCl2, 0.2 M NaCl, phosphate-buffered saline containing 0.05% sodium azide, and then stored at 4°C until use. The efficiency of the coupling reaction was determined by monitoring the A280 of the gel.

* This research was supported in part by Grant CA23365 from National Cancer Institute, Department of Health and Human Services. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used for nucleotides and polynucleotides are those of IUPAC-IUB Commission on Biochemical Nomenclature (CBN) 1970, J. Biol. Chem. 245, 5171-5176. Other abbreviations used are: RF, replicative form; BSA, bovine serum albumin; IgG1, immunoglobulin γ subtype 1; kDa, kilodalton; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.

14679
effluent from the column and was greater than no absorbent resins used in this study.

### Methods

**Enzyme Assays**—DNA polymerase activity was measured in a 250-μl reaction containing 0.04 M potassium phosphate buffer at pH 7.0, 0.2 mg/ml of activated calf thymus DNA, 1 mM dithiothreitol, 8 mM MgCl₂, 0.1 mM each of dATP, dCTP, dGTP, and [CH₃-3H]dTTP (100 counts/min/pmol), and 100 μg/ml BSA. Incubation was carried out at 35 °C, and aliquots (30 to 50 μl) were removed at various times and washed up on glass-fiber filters for acid-insoluble products. One unit of enzyme is defined as the amount that catalyzes the incorporation of 1 nmol of total dNTP into acid-insoluble material in 60 min at 35 °C.

DNA primase activity was measured in a 250-μl reaction containing 0.05 M Tris-HCl buffer at pH 8.0, 0.1 mM poly(dT), 1 mM dithiothreitol, 0.2 mM [γ-32P]ATP at 1000 to 5000 cpm/pmol, 8 mM MgCl₂, and 100 μg/ml BSA. Incubation was carried out at 35 °C, and aliquots (30 to 50 μl) were removed and processed on DE-81 paper (Whatman) and counted under double-isotope conditions in a liquid scintillation counter (15). One unit of DNA primase activity is defined as the amount that catalyzes the incorporation of 1 nmol of ATP into products in 60 min at 35 °C.

When single-stranded circular phage DNAs were used, typical reactions (200 to 400 μl) contained 0.04 M potassium phosphate at pH 7.5, 8 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM each of dC, dG, dT, and [8-3H]dATP at 300 to 400 cpm/pmol, 0.2 mM each UTP, CTP, GTP, and [γ-32P]ATP or [γ-33P]ATP at 1000 to 5000 cpm/pmol, 10 μg/ml DNA, and 100 μg/ml BSA. When [32P]ATP was used in the reaction the concentration of ATP was 1 mM. Products formed in the reaction were processed on DE-81 paper as described above.

**Purification of DNA Polymerase-a from Calf Thymus Glands**—All operations were carried out at 4 °C. Frozen calf thymus glands (440 g) were homogenized in a Waring blender in 1760 ml of 0.04 M potassium phosphate at pH 7.4, 0.04 M NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1% dimethyl sulfoxide (Buffer A). A cloudy supernatant solution was obtained after centrifugation of the homogenate for 15 min at 4500 rpm in an H4000 rotor in a Sorvall 3B centrifuge. After filtering through 4 layers of cheesecloth, 71 ml of 3% protamine sulfate solution were added with stirring. Stirring was continued for 15 min, and then the suspension was allowed to settle for 15 min. The supernatant solution obtained after centrifugation at 8500 rpm for 30 min in a GS-3 rotor in a Sorvall 5B centrifuge still showed slight turbidity and was clarified by addition of 10% Nonidet P-40 to a final concentration of 0.1%.

The clarified protamine sulfate supernatant (1720 ml) was loaded directly onto a 5-ml mouse monoclonal antibody column containing 10 mg of mouse IgG₁ to calf thymus DNA polymerase-a at a flow rate of 4 ml/min. After the loading was complete, the column was washed with 250 ml of Buffer A, 50 ml of 1 M NaCl in 0.05 M Tris-HCl buffer at pH 8.0, followed by 50 ml of 3 M NaCl in 0.05 M Tris-Cl buffer at pH 8.0. Protein remaining on the column was eluted with 3.2 ml of 2 M MgCl₂ buffered with 0.05 M Tris-HCl at pH 8.0. Greater than 95% of the DNA polymerase and DNA primase activity adsorbed were found by direct assay of the 3.2 M MgCl₂ elute. This fraction was desalted on a Sephadex G-50 column (2.3 x 60 cm) equilibrated with 0.05 M NaCl in 0.05 M Tris-HCl at pH 8.0 and 10% glycerol. Bovine IgG, present in calf thymus extracts and bound to the unsaturated Protein A sites on the Sepharose, was removed by passing the Sephadex G-50 fraction through a 5-ml Protein A-Sepharose column equilibrated with 0.05 M NaCl in 0.05 M Tris-HCl at pH 8.0 and 10% glycerol. After the Protein A-Sepharose treatment, the fraction was dialyzed against 25 mM potassium phosphate at pH 7.4, 0.2 mM dithiothreitol, and 50% glycerol. Activities were found to be stable during storage for 2 months at −20 °C.

**Gel Electrophoresis**—Peptides present in the purified calf thymus DNA polymerase-a fraction were analyzed on SDS-polyacrylamide gels prepared as described by Laemmli (16) using a 6% stacking gel and a 10% separating gel.

Agarose gel electrophoresis of DNA samples was carried out on 1% gels at neutral pH as previously described (17). After visualization of DNA bands by staining with ethidium bromide, the gel was dried onto a sheet of DE-81 paper and autoradiographed on Kodak X-Omat AR film at −70 °C with a DuPont Cronex Lightning Plus intensifying screen. Restriction fragments of DNA samples were analyzed on 6% polyacrylamide gels run in 40 mM Tris acetate buffer at pH 8.0 containing 2 mM EDTA. RNA primers synthesized by the calf thymus enzyme was analyzed on urea-polyacrylamide sequencing gels containing 20% acrylamide, 7.0 M urea, 0.09 M Tris borate at pH 8.3 and

---

**Table I**

| Fraction                        | Volume | Protein | Specific activity | Total enzyme activity |
|---------------------------------|--------|---------|-------------------|-----------------------|
| Crude extract*                 | 1,780  | 24,700  | 21.4              | 529,000               |
| Protamine sulfate supernatant  | 1,720  | 14,600  | 35.0              | 511,000               |
| MgCl₂ eluate from Mab column†  | 17     | 10.7    | 32,000            | 342,000               |
| Sephadex G-50                  | 28     | 10.0    | 27,300            | 273,000               |
| Protein A Sepharose            | 33     | 2.2     | 105,000           | 231,000               |

*Crude extract was made from 440 g of frozen calf thymus glands.
†About 30% of DNA polymerase activity present in the protamine sulfate supernatant did not bind to the mouse monoclonal antibody (Mab) column.
FIG. 2. Sucrose gradient analysis of calf thymus DNA polymerase and primase activities. Purified enzyme fraction was concentrated by dialysis against solid Sephadex G-50, dialyzed against 1.5 M NaCl in 50 mM potassium phosphate at pH 7.4, 1 mM dithiothreitol in 10% glycerol overnight followed by dialysis against 0.5 M NaCl in 50 mM potassium phosphate at pH 7.4, 1 mM dithiothreitol, and 10% glycerol. Recovery of DNA polymerase activity was greater than 80% after the concentration and dialysis treatments. The dialyzed sample was loaded directly onto a linear 5 to 20% (w/v) sucrose gradient made up in 0.5 M NaCl in 50 mM potassium phosphate at pH 7.4, 1 mM dithiothreitol, and 10% glycerol. Centrifugation was carried out for 24 h at 4 °C in a SW 50.1 rotor in a Beckman ultracentrifuge. Standard proteins, ovalbumin and aldolase, were analyzed on separate gradients. At the end of centrifugation, gradients were fractionated with a Buchler Densi-Flow apparatus into 39 equal volume fractions with fraction 1 representing the top of the gradient. Enzyme assays were carried out using 5 μl of each gradient fraction in 55-μl reaction mixtures set up as described under “Methods.” For activated DNA primed reactions, [CH3-3H]dTTP was used, and incubation time was 7.5 min. For 6X174 DNA primed reactions, [8-3H]dATP was used, and incubation was for 30 min. For poly(dT) replication, [8-3H]dATP and [α-32P]ATP were used, and incubation was for 2 h. Recoveries of DNA polymerase and DNA primase activities on the sucrose gradient were greater than 70%.

RESULTS

Purification of Calf Thymus DNA Polymerase-α—A summary of the calf thymus DNA polymerase-α purification procedure using a monoclonal antibody column is presented in Table I. This procedure for preparation of DNA polymerase-α from calf thymus glands outlined under “Experimental Procedures” has several advantages. First, the entire procedure takes 2 days. Second, the enzyme isolated is fully active. Third, recovery of the total enzyme activity is good. Fourth, the capacity of the antibody column is high, since up to 3 mg of fully active mouse monoclonal antibody can be covalently coupled to each ml of Protein A-Sepharose. Finally, the procedure does not destroy the immunoabsorbent resin, and the column can be reused.

In the purification of DNA polymerase-α from calf thymus glands an additional step must be included to remove endogenous IgG in the thymus extracts. This simply requires passing the fraction through a Protein A-Sepharose column. A similar procedure, originally developed for purification of human terminal deoxynucleotidyltransferase from human lymphoblastoid cells, provides rapid preparation of homoge-
eluate when the column was made with antibody 42 and only about 35% when antibody 17 was used. SDS-polyacrylamide gel analysis of the peptides as well as protein measurements on the fraction eluted from antibody 17 column showed good recovery of protein, but inactivation of enzyme activity appears to have taken place.

The differences in binding capacity and recovery of enzyme activity from the immunoabsorbent column must be related to the affinity of antibody as well as the specific nature of the antibody-antigen interaction. All four mouse monoclonal antibodies tested in this study are high-affinity antibodies as indicated by the stability of antigen-antibody interaction in 3 mM NaCl, yet the total recoveries of enzyme and binding capacities varied. In order to develop an efficient process for protein purification purposes, it may be important to compare a number of different monoclonal antibodies.

Concentrated MgCl₂ solutions appear to provide effective but mild antigen-antibody dissociation. When similar procedures are used in the purification of low-molecular-weight terminal transferase from immunoabsorbent columns, activity recovery was near 100% when eluted with unbuffered 1 mM MgCl₂ solution. With calf thymus DNA polymerase-α, the recoveries of enzyme activity were 2 and 0% when unbuffered MgCl₂ solution was used for antibody 42 column and antibody 17 column, respectively. Enzyme activity recoveries were greatly improved when the MgCl₂ solution was buffered with Tris-HCl at pH 8.0. The concentration of MgCl₂ as well as the pH of the solution to be used to elute protein from the column should be determined for effective operation of this kind of immunoabsorbent columns.

The use of MgCl₂ for dissociation of the antigen and antibody complexes on the immunoabsorbent resin made by covalent coupling of antibody to Protein A-Sepharose also allows indefinite reuse of the resin. We have used a mouse monoclonal antibody coupled to a Protein A-Sepharose column for over 50 purifications of calf thymus terminal transferase. The column still retains greater than 50% of the original binding capacity.

Table I shows a summary of purification of calf thymus DNA polymerase-α on the antibody 42 column. The overall recovery of enzyme activity exceeds 40%. The clarification of the crude extract appears to be important for successful purification of the enzyme. Crude extracts were carefully titrated with protamine sulfate to obtain maximum removal of nucleic acids and minimum loss of enzyme activity. For a 20% homogenate of calf thymus glands, we found 0.12% protamine sulfate provided the best results. The small amount of turbidity remaining after the protamine sulfate treatment was eliminated by addition of a small amount of nonionic detergent Nonidet P-40. The major loss in enzyme activity occurs during loading of the protamine sulfate supernatant onto the antibody column since about 30% of the activity is found in the flowthrough. Over 90% of enzyme activity bound to the immunoabsorbent resin is recovered in the MgCl₂ eluate. A small amount of activity, less than 0.5% of DNA polymerase and DNA primase activities, was found in the 1 M NaCl wash. Lane A in Fig. 1 shows the SDS peptides present in the purified calf thymus DNA polymerase fraction. Four major peptides (160, 68, 55, and 48 kDa) and two minor peptides (185 and 40 kDa) are present in this fraction. Lane B of Fig. 1 shows the SDS peptides present in the 1 M NaCl wash fractions. Since the 55-, 48-, and 40-kDa peptides are

![Fig. 3](image-url)
Fig. 4. Kinetics of replication as a function of phage fd DNA concentration. Reaction mixtures contained 1 pg of polymerase-primase fraction in 200 μl as described under "Methods" and in the legend to Fig. 3 with [8-3H]dATP and various concentrations of fd DNA. At various times of incubation at 35 °C, 25-μl samples were withdrawn and processed on glass-fiber paper for acid-insoluble products.

TABLE II
Requirements of calf thymus DNA polymerase-primase complex in replication of φX174 DNA and poly(dT)

| Reaction condition | Activity | Reaction conditions | Activity |
|--------------------|----------|---------------------|----------|
| φX174 DNA          | 100      | Complete            | 100      |
| -ATP               | 38       | -Poly(dT)           | 0        |
| -GTP               | 53       | +E. coli PolI       | 199      |
| -CTP               | 80       | +E. coli PolI, -poly(dT) | 0      |
| -UTP               | 83       | +E. coli PolI, -ATP | 0        |
| -ATP, GTP          | 7        |                     |          |
| -ATP, UTP          | 28       |                     |          |
| -CTP, CTP          | 27       |                     |          |
| -GTP, UTP          | 38       |                     |          |
| -CTP, UTP          | 53       |                     |          |
| -ATP, GTP, CTP     | 7        |                     |          |
| -GTP, CTP, UTP     | 13       |                     |          |
| -ATP, GTP, UTP     | 15       |                     |          |
| -ATP, GTP, UTP     | 6        |                     |          |

found in both fractions and the 1 M NaCl fraction is not active, we believe that the unique peptides (185, 160, and 68 kDa) must be responsible for the DNA polymerase and DNA primase activities. Earlier experiments suggest that the 160-kDa peptide is DNA polymerase. The lower-molecular-weight peptides, certainly tightly bound either directly to the column resin or to bound proteins, may represent degraded enzyme peptides or modulating peptides in the enzyme complex.

The DNA polymerase-α fraction isolated from the immunoabsorbent column contains DNA primase activity that can be measured directly by incorporation of [α-32P]ATP in a poly(dT) primed reaction, showing a final specific activity of 747 units/mg in the complex (Table I). The presence of DNA primase in immunoabsorbent-purified DNA polymerase fraction suggests that these enzymes are present as a complex in the crude extract (8, 18). Estimation of the molecular weight of the complex would be helpful in understanding peptide stoichiometry.

An unacceptable degree of aggregation of the protein in the purified DNA polymerase-primase fraction was detected by sucrose gradient analysis of the final purified fraction. Addition of NaCl to 0.5 M did not disaggregate these fractions. In 3 L. M. S. Chang and P. Plevani, unpublished results.
order to obtain some information on the native molecular weight of the enzyme complex, the fraction was first treated with 1.5 M NaCl and then dialyzed into 0.5 M NaCl before analysis on a sucrose gradient made up in 0.5 M NaCl. The enzyme activity profile on the sucrose gradient is presented in Fig. 2. DNA polymerase activity, assayed with activated DNA, sedimented as a double peak at 7.4 and 9.3 S corresponding to molecular weights of about 158,000 and 223,000, respectively. DNA primase activity, assayed directly by \([\alpha-\text{\textsuperscript{32}}P]\)ATP incorporation in poly(dT) replication or \(\phi X174\) DNA replication, is found exclusively in the faster sedimenting species. These results suggest that the immunoabsorbent column-purified DNA polymerase-primase complex consists of free DNA polymerase together with a DNA polymerase-primase complex. Free native DNA polymerase is about 160 kDa, and the polymerase-primase complex appears to be 223 kDa. One possible explanation is that the calf thymus DNA polymerase-primase complex we have detected consists of the 160- and the 68-kDa peptides. The lower-molecular-weight peptides do not appear to be specific to the complex, and the 185-kDa peptide is present in trace amount only. We would prefer to postpone speculation about the relation of these peptides to the complex structure.

Properties of Calf Thymus DNA Polymerase-DNA Primase—Synthesis catalyzed by the purified calf thymus enzyme was completely dependent on added template. With circular single-stranded phage DNA as template, maximum synthesis is dependent on the presence of all 4 rNTPs, although omission of pyrimidine ribonucleoside triphosphates decreased synthesis by only 50% (Table II). ATP is required for the replication of poly(dT). Addition of 100 units/ml of \(E.\ coli\) DNA polymerase I stimulated the reaction rate only about 2-fold. In contrast, poly(dT) replication catalyzed by the yeast DNA polymerase-primase complex was found to be stimulated 7- to 8-fold by added \(E.\ coli\) DNA polymerase I (18). The results obtained with the calf thymus enzyme suggest that the polymerase and primase activities are more tightly coupled than the yeast enzyme complex.

RNA primers synthesized in the replication reaction can be demonstrated by direct incorporation of ribonucleotides into reaction products. Fig. 3 shows the time courses of poly(dT) and \(\phi X174\) DNA replication. When dNTPs were left out of the reaction mixtures the incorporation of \([\alpha-\text{\textsuperscript{32}}P]\)AMP in the poly(dT) reaction was linear until greater than 50% of avail-
able template was utilized. We have utilized this property of the calf thymus enzyme to measured primase activity directly (Table 1). When dATP was included in the replication reaction, incorporation of [α-32P]AMP is depressed about 4-fold. The rate of incorporation of AMP in φX174 DNA replication is less than 1% of that found in poly(dT) replication, and the net incorporation of AMP is also depressed 2- to 3-fold when dNTPs were present in the reaction. Similar experiments on φX174 DNA replication using [γ-32P]ATP show that less than 4 molecules of [γ-32P]ATP are incorporated per DNA molecule after complete replication (data not shown).

The calf thymus DNA polymerase-primase has a very high affinity for template DNA. The incorporation curves in Fig. 4 demonstrate that the enzyme is nearly saturated at 1 μg/ml of φX174 DNA. After 60 min of incubation, replication of 0.16 and 0.8 μg/ml of phage DNA were 75 and 81%, respectively (Fig. 4). At higher concentrations of DNA replication continued until near completion. Template saturation measurements estimate the Km of the calf thymus DNA polymerase-primase for fd DNA to be 0.36 μg/ml (data not shown).

Although the calf enzyme complex replicates heat-denatured calf thymus DNA (data not shown) and single-stranded circular phage DNA efficiently, the enzyme complex carried out only limited synthesis on double-stranded DNAs. When native calf thymus, φX174 RF I, SV40, and herpes simplex type I DNAs were used as templates, an initial synthesis (amounting to less than 2% replication) was observed, but the reactions did not progress (data not shown). This initial synthesis may represent replication of single-stranded regions in these DNA samples.

Products of Synthesis Catalyzed by the Calf Thymus DNA Polymerase-Primase—Incorporation experiments demonstrate that replication of φX174 DNA by calf thymus polymerase-primase proceeds to completion, suggesting that the entire phase DNA molecule might be replicated by the enzyme complex. Fig. 5 shows the time course of appearance of HaeIII fragments in the products of φX174 DNA replication. When replication was greater than 50% (lanes D and E), all of the HaeIII restriction fragments were detected in the product, proving that template molecules are fully replicated.

The principal product of φX174 DNA replication catalyzed by the calf polymerase-primase complex is nicked circular DNA (Fig. 6A). Single-stranded circular phage DNA is quantitatively converted to a double-stranded form as illustrated by ethidium bromide staining of the products of the replication reaction after separation by electrophoresis on 1% agarose. In addition to the major population of nicked circular form, a minor population of double-stranded linear molecules is also present. The double-stranded linear molecules are probably produced from single-stranded linear DNA molecules present in the φX174 DNA used as template. The RNA primers associated with the products when the reaction was carried out in the presence of [α-32P]ATP (lanes a and a') and in the presence of [γ-32P]ATP (lanes b and b') are shown in the autoradiograms in Fig. 6B. Because of the small amount of [γ-32P]ATP incorporation in φX174 DNA replication products, we thought that the initiation sites on φX174 DNA might be specific sequences. When the [γ-32P]ATP labeled DNA products were cleaved with HaeIII and analyzed by gel electrophoresis, radioactivity was found to be associated with single and double fragments (data not shown) suggesting no site-specific initiation.

We attempted to analyze the size of the RNA primers synthesized in the replication reaction after isolation of the reaction products by phenol treatment followed by ethanol precipitation. The major problem encountered in this analysis is the poor recovery of the RNA products. This is probably due to solubility of short oligonucleotides in ethanol. In order to examine the total populations of RNA primers synthesized in the reaction, we carried out the DNase I digestion directly on the reaction mixtures at the end of the replication reaction and analyzed [α-32P]AMP-labeled products on a DNA-sequencing gel. Reaction products of poly(dT) and φX174 DNA replications, with and without DNase I treatment, were loaded on the DNA-sequencing gel twice at 30 min apart, and the autoradiogram of the gel is presented in Fig. 7. The RNA products in poly(dT) reactions containing only ATP exhibit three populations, averaging about 20 nucleotides, 10 nucleotides, and 3 to 4 nucleotides (lanes A and A'). Treatment of these RNA products with DNase I did not significantly alter the size distribution (lanes B and B'). The RNA products synthesized in poly(dT) replication when dATP was present also showed three size distributions, averaging about 16 nucleotides, 3–4 nucleotides, and polymer form (lanes C and C'). When these poly(dT) replication products were digested with DNase, the oligoriboadenylates produced belong to two size distributions, 9–10 nucleotides and 3–4 nucleotides (lanes D and D').
### Table

| Template | Poly (dT) | φX174 DNA | Poly (dT) | φX174 DNA |
|----------|-----------|------------|-----------|------------|
| dNTPs    | - - + +   | - - + +    | - - + +   | - - + +    |
| DNase I  | - - - +   | - - - +    | - - - +   | - - - +    |

### Figures

![Urea-polyacrylamide gel](image)

**Fig. 7.** Analysis of RNA primers produced by calf thymus DNA polymerase-primase in the replication of poly(dT) and φX174 DNA. Reaction mixtures (100 µl) were as described under “Methods” except that 25 mM Hepes buffer at pH 7.5 was used for both poly(dT) and φX174 DNA replication reactions, and 50 µg/ml of φX174 DNA were used. Both [8-3H]dATP and [α-32P]ATP were present as labeled isotopes. After 2 h of incubation at 35 °C, half of each reaction (50 µl) was removed and incubated with 15 µg/ml of RNase-free pancreatic DNase I for 2 h at 35 °C, and the other half was kept in an ice bath. After the DNase I digestion, 20 µl of each reaction (with and without DNase I treatment) were spotted on a DE-81 filter and processed for polymer products formed. To the remainder of each sample, SDS and EDTA were added to 0.5% and 10 mM, respectively. The reaction mixtures were then diluted with an equal volume of 98% formamide, 10 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol. After further dilution with 49% formamide, 5 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol, the samples were boiled for 5 min, and 2 µl of each diluted sample were loaded on the urea-polyacrylamide gel. Samples were loaded twice on the same gel at 30-min intervals with lanes A through H showing the results for the first load and A' through H' showing the second load. Electrophoresis was stopped when the bulk of [α-32P]ATP radioactivity was found in the electrophoresis buffer. Products on the gel were visualized by autoradiography. The analysis showed that the DNase I treatment removed greater than 98% of the [8-3H]dAMP found in the products. The ratios of [α-32P]AMP found in the products of the replication reactions were 1:4:14:44 for φX174 DNA plus rNTPs plus dNTP: φX174 DNA plus rNTP minus dNTP: poly(dT) plus ATP and dATP: poly(dT) plus ATP minus dATP. Product 32P counts loaded onto the DNA-sequencing gel were 3200, 3300, 2100, 1800, 1200, 1000, 600, and 500 for Lanes A and A', B and B', C and C', D and D', E and E', F and F', G and G', H and H'. The numbers on the sides of the figure show the positions of the standards on the sequencing gel. The markers were synthesized using d(pA)₆, d(pA₆₆, d(pA)₆₆, and d(pA)₁₂ as initiators in terminal transferase reactions using chain terminator [α-32P]cordycepin triphosphate as substrate. Radioactivity on the autoradiogram in Lanes E', F', G', and H' below a chain length of 5 came from [α-32P]ATP.
dependent, showing rapid initiation and closely coupled DNA replication products (lanes C and C') may represent primer with a few deoxyadenylate residues. The results and interpretation of the primer sizes in poly(dT)-primed reactions obtained in this study are consistent with those observed with the mouse primase and the human KB cell DNA polymerase-primase (10, 19).

Discrete RNA primers detected in φX174 DNA reactions are 10 nucleotides or shorter when dNTPs were omitted from the reaction mixture (Fig. 7, lanes E and F') and 8 nucleotides or shorter when dNTPs were included (lanes G and G'). No discrete larger species were detected in φX174 DNA reactions. Treatment of φX174 reaction products with DNase I did not affect the size distribution of RNA primers (lanes F, H, F' and H') suggesting the absence of deoxyribonucleotides in the primers synthesized by the calf enzyme complex.

**DISCUSSION**

Calf thymus was the original source of material for large-scale purification of eukaryotic DNA polymerase-α (20), leading to the demonstration of the high-molecular weight (21), oligoribonucleotide initiation (22), and absence of associated exonucleases (23). Traditional methods of purification (1, 4, 24) demonstrated multiple species of DNA polymerase-α, and interpretation of the basis of the heterogeneity observed has not been possible.

The existence of DNA primase, providing oligoribonucleotide initiation for DNA synthesis, has now been demonstrated in several eukaryotic systems (8, 18, 19, 25-28) following the lead established by genetic and biochemical studies on *E. coli* DNA polymerase III (29). The DNA primase presumably occurs as some sort of a complex with DNA polymerase-α, but genetic analysis is not easily available in eukaryotic systems.

Use of monoclonal antibody purification now provides the possibility for more extensive analysis of the proteins participating in the DNA replication process in complex cells. The multipetide complex we have isolated in the current work is a good source of antigen for generating new antibody panels.

The monoclonal antibody columns used in this study should also permit examination of cell-extraction procedures with the intent of devising conditions that will allow isolation of complexes more approaching the true biological state. Complexes of this kind should provide a clearer picture of peptide stoichiometry. Through use of immunoaffinity purification, the analysis of DNA replication in eukaryotic systems at the molecular level now appears to be resolvable.

**REFERENCES**

1. Holmes, A. M., Hesselwood, I. P., and Johnston, I. R. (1976) *Eur. J. Biochem.* 62, 229-235
2. Fisher, P. A., and Korn, D. (1977) *J. Biol. Chem.* 252, 6528-6535
3. Kaguni, L. S., Rossignol, J., Conway, R. C., and Lehman, I. R. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 2221-2225
4. Masaki, S., Kowai, O., and Yoshida, S. (1982) *J. Biol. Chem.* 257, 7172-7177
5. Yamaguchi, M., Tanabe, K., Takahashi, T., and Matsukage, A. (1982) *J. Biol. Chem.* 257, 4484-4489
6. Chen, Y.-C., Bohn, E. W., Planck, S. R., and Wilson, S. H. (1979) *J. Biol. Chem.* 254, 11678-11687
7. Tanaka, S., Hu, S.-Z., Wang, T.-S.-F., and Korn, D. (1982) *J. Biol. Chem.* 257, 8386-8390
8. Hu, S. Z., Wang, T. S.-F., and Korn, D. (1984) *J. Biol. Chem.* 259, 1854-1864
9. Wahl, A. F., Kowalski, S. F., Harwell, L. W., Lord, E. M., and Bambara, R. A. (1984) *Biochemistry* 23, 1895-1899
10. Wang, T. S.-F., Hu, S. Z., and Korn, D. (1984) *J. Biol. Chem.* 259, 2902-2909
11. Kunkel, J. A., and Loeb, L. A. (1981) *Science (Wash. D. C.)* 213, 765-766
12. Chang, L. M. S., and Bollum, F. J. (1971) *Biochemistry* 10, 536-542
13. Bollum, F. J. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G., and Davis, D., eds) p. 592, Harper and Row, New York
14. Bollum, F. J., Augl, C., and Chang, L. M. S. (1984) *J. Biol. Chem.* 259, 5848-5850
15. Meade, C. G. (1964) *Proc. Natl. Acad. Sci. U. S. A.* 52, 1482-1488
16. Laemmli, U. K. (1970) *Nature (Lond.)* 227, 680-685
17. Sugden, B., Detryo, B., Roberts, R. J., and Sambrook, J. (1975) *Anal. Biochem.* 68, 30-46
18. Plevani, F., Badaracco, G., Augl, C., and Chang, L. M. S. (1984) *J. Biol. Chem.* 259, 7592-7609
19. Tseng, B. Y., and Aschen, C. H. (1982) *J. Biol. Chem.* 257, 7280-7283
20. Yoneda, M., and Bollum, F. J. (1965) *J. Biol. Chem.* 240, 3385-3391
21. Bollum, F. J. (1975) *Nucleic Acids Res. Mol. Biol.* 15, 109-144
22. Chang, L. M. S., and Bollum, F. J. (1972) *Biochem. Biophys. Res. Commun.* 46, 1354-1360
23. Chang, L. M. S., and Bollum, F. J. (1973) *J. Biol. Chem.* 248, 3386-3404
24. Glommi, F., Wahl, G., Jantzen, H., Hamprecht, K., Huber, U., and Kuenzle, C. C. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 6081-6085
25. Conway, R. C., and Lehman, I. R. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 79, 2523-2527
26. Shiohara, T., Neshon, E. M., Bayne, M. L., and Benbow, R. M. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 79, 7209-7217
27. Yagura, T., Kozu, T., and Seno, T. (1982) *J. Biol. Chem.* 257, 11211-11217
28. Huber, U. (1983) *EMBO J.* 2, 133-136
29. Kornberg, A. (1980) *DNA Replication*, W. H. Freeman and Co., San Francisco.