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Immunoaffinity-Purified DNA Polymerase α Displays Novel Properties†

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ABSTRACT: The purification and characterization of a novel and more intact form of the DNA polymerase α–primase complex from calf thymus are described. The polymerase–primase was enriched 10 000-fold to apparent homogeneity by chromatography on phosphocellulose, heparin–Sepharose, and an immobilized anti-human DNA polymerase α monoclonal antibody [SJK287-38; Tanaka, S., Hu, S., Wang, T. S.-F., & Korn, D. (1982) *J. Biol. Chem. 257*, 8386–8390]. A quantitative elution from the antibody column was achieved by shifting the pH from neutrality to between 12.5 and 13. From 1 kg of calf thymus, the procedure yields 1–2 mg of polymerase–primase with a specific activity of 30 000–40 000 units/mg for the polymerase and 15 000–20 000 units/mg for the primase. The complex sediments at 9 S through a sucrose gradient and exhibits a Stokes radius of 6.0 nm, yielding a native molecular mass of 335 000. Denaturing gel electrophoresis of the complex gives bands of M_rs 180 000, 155 000, 148 000, 73 000, 59 000, and 48 000 with a relative abundance of the two smallest subunits. Primase activity was partially resolved from the complex by centrifugation through sucrose gradients. The primer-forming activity was found to be associated with the M_r 59 000 and 48 000 polypeptides. In contrast to conventional preparations, the immunopurified polymerase displays several features which show it is the most intact form of the enzyme known to date. The deoxynucleoside triphosphate K_m values are all within the range of 0.6–0.9 μM. The K_m for binding to a single RNA primer on M13 DNA is 3.5 nM; the K_i for nonspecific binding to unprimed DNA is 70 μM (nucleotide). The polymerase–primase converts single-stranded M13 DNA into the double-stranded form within 10–30 min. During this process, 3–10 RNA primers are formed. With singly RNA-primed M13 DNA, the complex exhibits a maximal rate of DNA synthesis of 26 nucleotides/s. Both KCl and potassium acetate stimulate DNA synthesis on activated DNA 2–3-fold at concentrations of 90–150 and 120–180 mM, respectively, and on self-initiated M13 DNA 1.3–2-fold at concentrations of 60–90 and 60–120 mM, respectively. Hence, this immunoaffinity-purified form of polymerase–primase maintains many of the properties which are characteristic of in vivo function.

The purification of DNA polymerase α, the main replicative entity in higher eukaryotes, is one of the most difficult tasks in preparative biochemistry. Although initial attempts to purify this enzyme date back nearly 3 decades (Bollum, 1960), the subunit structure, the enzymatic features, and its association with accessory proteins are still under debate. Most likely, it is the vulnerability to proteolytical degradation that is responsible for the broad heterogeneity of the isolated α-polymerases that have been obtained in different laboratories [for recent reviews, see Campbell (1986) and Fry and Loeb (1986)]. However, in the past 3 years, the picture has become clearer, and a uniform enzyme has been isolated by the use of immunoaffinity purification schemes utilizing immobilized monoclonal antibodies to DNA polymerase α (Wahl et al., 1984; Chang et al., 1984; Wang et al., 1984; Holmes et al., 1986; Wong et al., 1986; Zahradka, 1987). There is now a general agreement that DNA polymerase α [and also the analogous yeast enzyme DNA polymerase I; see e.g., Plevani et al. (1985)] typically consists of three to five subunits, sediments at 9 S through sucrose gradients, and displays both DNA polymerase and DNA primase activities. Thus, the structure and properties of the immunoaffinity-purified enzymes are similar to the 9–10S forms that have been isolated earlier by conventional column chromatography (Banks et al., 1979; Grosse & Krauss, 1981, 1985; Masaki et al., 1982; Kaguni et al., 1983a).

We report here our purification of a DNA polymerase α–primase complex from calf thymus by employing an immunoaffinity protocol that uses a commercially available monoclonal antibody. Originally, the protocol was designed to purify large amounts of the individual subunits of the complex that in turn could be used for further antibody production and without regard to the isolated enzymatic activity. However, we achieved a quantitative elution of DNA polymerase α (pol-α)[1] polypeptides by eluting the antibody column with 0.1 M K_3PO_4 (pH 12.5–13) and found that the enzyme was surprisingly active so long as the eluate was neutralized immediately after running the column. The polymerase–primase isolated in this way displays many properties that are similar to those that must exist for in vivo function but which have not been described so far, neither for conventionally purified enzymes nor for immunoaffinity-purified ones. These in vivo like properties are low K_ms for dNTPs and primers, a salt optimum that comes close to that observed inside the cell, an approximately 3–5-fold faster replication of natural single-stranded DNA as compared to hitherto known preparations, and an elongation rate on M13 single-stranded DNA

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[1] Abbreviations: BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; dNMP, deoxynucleoside monophosphate; dNTP, deoxynucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin γ; kDa, kilodalton(s); $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; pol-α, DNA polymerase α; Tris, tris(hydroxymethyl)aminomethane.

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that approaches the in vivo rate of replicational fork migration. We also describe a method for the separation of the primase activity from the polymerase–primase complex. This allows us to assign the M_r 48 000 and 59 000 subunits to the primase function.

Materials and Methods

Materials. DEAE-cellulose (DE52) and phosphocellulose (P11) were from Whatman. Heparin–Sepharose was prepared by coupling 2.5 g of heparin (Serva, 169 units/mg, or Sigma, grade I) to 400-mL packed volume of BrCN-activated Sepharose 4B-CL (Pharmacia). Unlabeled rNTPs and dNTPs were from Boehringer Mannheim. [α-^{32}P]dATP was from Amersham. M13 DNA was prepared as described (Grosse & Krauss, 1984). Singly ori-primed M13 DNA and multiply RNA-primed M13 DNA were produced by the method of Villani et al. (1981). Activated DNA was prepared as described by Aposhian and Kornberg (1963). Poly(dC,dT), poly(dA-dT), poly(dA), and $(dT)_{10}$ were from PL–Pharmacia. *Eco*RI-digested SPP1 DNA and *Hin*dIII-digested λ DNA were a kind gift of A. Manns. Dephosphorylation and 5′ labeling was according to Maniatis et al. (1981). DNA polymerase I (large fragment) was a kind gift of F. Eckstein, this department. Biochemically purified polymerase–primase was prepared to a specific activity of 40 000 units/mg as described by Grosse and Krauss (1981). The hybridoma line SJK287-38 (Tananka et al., 1982) was obtained from the American Type Culture Collection (ATCC No. CRL 1644).

Enzyme Assays. Pol-α activity was measured in a 50-μL reaction containing 20 mM Tris–acetate, pH 7.3, 75 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM each of dGTP, dCTP, dTTP, and [α-^{32}P]-dATP (10 cpm/pmol), and 100 μg/mL BSA. Either 0.2 mg/mL activated DNA or 50 μM (nucleotide) single-stranded M13 DNA served as a template. Incubation was carried out at 37 °C. One unit of enzyme is defined as the amount that catalyzes the incorporation of 1 nmol of dAMP into acid-insoluble material in 1 h at 37 °C with activated DNA as a template-primer.

The primase assay contained 20 mM Tris–acetate, pH 7.3, 10 mM magnesium acetate, 100 μM (nucleotide) poly(dC,T), 50 μM dGTP, 50 μM [α-^{32}P]dATP (30 cpm/pmol), 1 mM ATP, 0.1 mM GTP, 1 mM dithiothreitol, 100 μg/mL BSA, and 25 units/mL DNA polymerase I (large fragment) in a total volume of 10 μL. One unit of primase is defined as the amount that leads to an incorporation of 1 nmol of dNMP/h at 37 °C (Grosse & Krauss, 1985).

Protein Determination. Protein concentration was determined by the method of Bradford (1976) with lysozyme used as the standard.

Electrophoresis. $NaDodSO_4$–PAGE was carried out in a 10% slab gel with a 5% stacking gel by the method of Laemmli (1970). Densitometrical scanning was done with a "high-performance" gel scanner (Kronberg et al., 1984). Agarose gel electrophoresis was performed as described (Grosse & Krauss, 1984).

Sucrose Density Gradient Centrifugation. Ultracentrifugation was carried out at 4 °C in a Beckman SW56 Ti rotor at 40 000 rpm for 16 h. A linear 5–20% (w/v) sucrose gradient was formed in 30 mM potassium phosphate, pH 7.8, 0.5 M KCl, 10 mM $Na_2S_2O_5$, 1 mM EDTA, 7 mM 2-mercaptoethanol, and 0.8% (v/v) Ampholine (pH 3.5–10, LKB).

Gel Filtration. The Stokes radius of pol-α was determined on a Sepharose 6B column (1.77 cm² × 75 cm) equilibrated with 30 mM potassium phosphate, pH 7.8, 0.5 M KCl, 10 mM $Na_2S_2O_5$, 1 mM EDTA, and 7 mM 2-mercaptoethanol. Thyroglobolin (8.5 nm), urease (6.1 nm), catalase (5.2 nm), and BSA (3.5 nm) were used as calibration standards. Blue dextran and 1 M KCl were used to determine V_0 and V_T, respectively.

Production of Monoclonal Antibodies. Cells of the hybridoma line SJK287-38 (Tanaka et al., 1982) were grown in 500-mL spinner cultures of Dulbecco's minimal essential medium (Gibco), containing 5% fetal bovine serum (Gibco), to a density of 2×10^6 cells/mL. Cells were removed by centrifugation (650*g*, 10 min). The supernatant was then clarified by centrifugation at 15000*g* for 20 min at 4 °C. To each milliliter of clarified supernatant, 300 mg of $(NH_4)_2SO_4$ was slowly added with constant stirring. The pH was maintained at 7.2 by the addition of 1 M NaOH. After 30 min of stirring at 4 °C, the precipitated proteins were collected by centrifugation (30000*g*, 30 min) and dissolved in a minimal volume of 20 mM potassium phosphate, pH 8.0. The ammonium sulfate was removed by extensive dialysis against 20 mM potassium phosphate, pH 8.0, and 40 mM KCl. Then the IgGs were loaded on a column of DEAE-Sepharose (2.4 cm² × 42 cm) that was equilibrated with the same buffer used in the dialysis. IgGs of better than 95% purity and free from proteases were obtained from the flow-through fractions. The antibodies were dialyzed against several changes of 0.1 M $NaHCO_3$ and 50% glycerol and stored at −20 °C until usage.

Immobilization of Monoclonal Antibodies. To remove the glycerol, the antibodies were dialyzed against 0.1 M $NaHCO_3$ and, if necessary, further concentrated by dialysis against 20% (w/w) poly(ethylene glycol) (PEG-20 000, Serva) in 0.1 M $NaHCO_3$ to a final concentration of about 4 mg/mL. BrCN-activated Sepharose 4B (Pharmacia) was pretreated as prescribed by the manufacturer. To 3.5 mL of BrCN-activated Sepharose (1 g dry weight), 10–12 mg of monoclonal antibody was added, and the mixture was shaken for 2 h at 4 °C. Binding was followed by measuring the absorbance at 280 nm of the supernatant. Typically, more than 90% of the antibodies bound to the column material during that time. To neutralize any free reactive groups, the material was shaken for another 2 h at room temperature with 6 mL of 1 M ethanolamine hydrochloride, pH 8.3. The material was poured into a column and washed with 300 mL of 0.1 M potassium phosphate, pH 7.8. Column material was stored at 4 °C in the presence of 0.01% NaN_3 for at least 6 months without any loss of binding capacity.

Immunoaffinity Purification of Calf Thymus DNA Polymerase α with Monoclonal Antibody SJK287-38. The following procedures were carried out at 4 °C. All potassium phosphate buffers used in this procedure also contained 1 mM EDTA, 7 mM 2-mercaptoethanol, 10 mM $Na_2S_2O_5$, 0.5 mM phenylmethanesulfonyl fluoride, and 1% (v/v) Trasylol (Bayer, Leverkusen, FRG). Thymus glands were obtained from the local abattoir and stored at −80 °C. Glands (1000 g) were allowed to thaw overnight and homogenized in 3 L of 50 mM Tris-HCl, pH 7.8, 25 mM KCl, 5 mM $MgCl_2$, 10 mM $Na_2S_2O_5$, 7 mM 2-mercaptoethanol, 250 mM sucrose, and 1% (v/v) Trasylol for 2 min at the low setting of a Waring Blendor. The material was passed through four layers of cheesecloth in order to remove undisrupted material, connective tissue, and lipid particles. Nuclei were removed by centrifugation at 1000*g* for 10 min. The supernatant was adjusted to pH 7.0 by the addition of H_3PO_4, and 300 mL of phosphocellulose (P11, Whatman, equilibrated in 30 mM potassium phosphate, pH 7.0) was added. The slurry was stirred for 30 min at 4 °C and then passed through a sintered glass funnel by applying water aspirator vacuum. The phospho-

cellulose was washed with 2 L of 70 mM potassium phosphate, pH 7.0, on the funnel by applying water aspirator vacuum. Proteins were eluted with 500 mL of 0.25 M potassium phosphate, pH 8.0, also under water aspirator vacuum. Approximately 100-mL fractions were taken; those containing pol-α activity were combined (about 300 mL) and diluted with distilled water to a conductivity equivalent of 70 mM potassium phosphate. To the diluted fractions, 250 mL of heparin–Sepharose was added and the slurry stirred for 30 min. Unbound material was removed by filtration through a sintered glass funnel under water aspirator vacuum. The heparin–Sepharose was washed on the funnel with 750 mL of 70 mM potassium phosphate, pH 7.8. The washed material was poured into a column (2.4 $cm^2 \times 42$ cm) on top of a 50-mL pad of fresh heparin–Sepharose. A 2000-mL gradient from 70 to 250 mM potassium phosphate, pH 7.8, was used to elute the column with a flow rate of about 200 mL/h. Pol-α activity eluted at about 130 mM potassium phosphate (Grosse & Krauss, 1981). The active fractions were combined, and the enzyme was precipitated by addition of 400 mg of $(NH_4)_2SO_4$ to 1 mL of liquid, followed by centrifugation at 15000g for 20 min. The pellet was then dissolved in a minimal volume of 30 mM potassium phosphate, pH 7.8, 1 mM 2-mercaptoethanol, and 1 mM EDTA and dialyzed against several changes of the same buffer. This dialyzed solution (10 mL) was then clarified by centrifugation (15000g, 20 min), and thereafter, approximately 1 mL (packed volume) of immobilized monoclonal antibody was added. Following shaking for 45 min, the material was poured into a small column (0.2 $cm^2 \times 5$ cm) and washed first with 50 mL of dialysis buffer and then with 50 mL of 50 mM Tris-HCl, pH 8.6, 150 mM KCl, and 1 mM 2-mercaptoethanol. The enzyme was eluted with 5 mL of 0.1 M potassium phosphate, pH 12.5–13, 1 M KCl, and 10% (v/v) glycerol. The inclusion of 1 M KCl is not mandatory but facilitates a quantitative elution from columns that had been used several times. Immediately after elution, the pH of each fraction was adjusted to 7–8 by the addition of KH_2PO_4, and dithiothreitol was added to a final concentration of 4 mM. Active fractions were combined (2–3 mL), dialyzed against 30 mM potassium phosphate, pH 7.8, 10 mM $Na_2S_2O_5$, 1 mM EDTA, 1 mM dithiothreitol, and 50% (v/v) glycerol, and stored at −20 °C. Polymerase and primase activities were stable for at least 12 months. A summary of the purification scheme is given in Table I.

Table I: Immunopurification of Polymerase–Primase from Calf Thymus[a]

step	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)
(1) crude extract	85700	300000	3.5	100
(2) phosphocellulose	870	130000	120	43
(3) heparin–Sepharose	180	90000	500	30
(4) antibody column	1.6	46000	29000	15

[a] The protein content and enzyme activity were determined as described under Materials and Methods. Purification was from 1000 g of tissue.

Results

Immunoaffinity Purification of DNA Polymerase α–Primase Complex with Monoclonal Antibody SJK287-38. During the last 3 years, several immunoaffinity purification protocols for mammalian DNA polymerases α (and yeast DNA polymerase I) have been reported. Of those, protocols that are based on commercially available antibodies or hybridoma lines are of particular interest. On the basis of the findings of Wahl et al. (1984) that the anti-human pol-α monoclonal antibody SJK287-38 (Tanaka et al., 1982) also neutralizes the calf thymus enzyme, we have developed a rapid, efficient, and highly reproducible protocol for the large-scale purification of an intact form of pol-α–primase from this organism. As described under Materials and Methods, calf thymus glands were disrupted under isotonic conditions, nuclei were removed, and the cytosolic extract was directly stirred into phosphocellulose. Pol-α activity was batchwise eluted and further enriched by heparin–Sepharose chromatography. Fractions with pol-α activity were combined and adjusted to 400 mg/mL ammonium sulfate. The precipitated proteins were dissolved in low-salt buffer and rotated top over bottom with SJK287-38 antibodies which were covalently coupled to BrCN-activated Sepharose 4B. More than 90% of pol-α activity bound to the immobilized antibody within 30 min. The antibody column was extensively washed with buffer containing 150 mM KCl. Typically, less than 3% of the pol-α activity leaches from the column during the washing procedure. Pol-α was eluted with unadjusted 0.1 M K_3PO_4 (pH 12.5–13; determined by "Alkalit pH 7.5–14" indicator sticks, Fa. Merck, Darmstadt, FRG). Fractions were neutralized immediately after elution by the addition of KH_2PO_4.

Comments on the Purification Scheme. We found it possible to isolate active pol-α from crude extracts without any prior enrichment step. However, those preparations displayed a great variability in their polypeptide pattern and were contaminated by proteases. Therefore, for reproducible results, we usually prepurify pol-α by batch concentration on phosphocellulose, and by chromatography on heparin–Sepharose. This procedure leads to pol-α preparations that can be stored for more than 1 year without any notable change in activity and subunit structure. However, if stability is not so critical, e.g., for the production of antigen, prepurification with only the phosphocellulose step might be sufficient.

About 50% of the pol-α activity was eluted from the antibody column by shifting to high pH between 12.5 and 13. Several other methods for eluting the enzyme were tried but found to be less effective. About 10–20% of the activity applied was eluted at pH 4.5 [as described by Wang et al. (1984)], but the acidic eluted activity was not stable. Less than 3% of the activity applied was eluted with various concentrations of KCl or NaCl or with pH 8 buffered and unbuffered solutions of 3.5 M $MgCl_2$, 50% (v/v) ethylene glycol, or 50% (v/v) glycerol.

Columns with BrCN-coupled antibodies were found to be stable. No problems arose when the columns were run in the presence of 1 mM 2-mercaptoethanol or treated for short periods of time at high pH (up to 13). To give an indication of the stability of the column, we have reused one BrCN-coupled antibody column 10 times, with less than a 20% loss in binding capacity.

Structure of the Immunoaffinity-Purified Pol-α–Primase Complex. From the given protocol, 1–2 mg of nearly homogeneous pol-α–primase complex is obtained in a 15% yield from 1 kg of calf thymus. The specific activity of pol-α is 30 000–40 000 units/mg, and the primase specific activity is 15 000–20 000 units/mg (Table I). A typical enzyme preparation contains a cluster of α-subunits with molecular weights of 180 000, 155 000, and 148 000, whose relative intensities vary somewhat from preparation to preparation. Furthermore, we always find polypeptides with molecular weights of 73 000, 59 000, and 48 000. The relative intensity of the α-subunit cluster to the intensities of the three smaller subunits also varied somewhat from preparation to preparation but on the average were roughly 1:1:2–4:2–4, assuming that all the

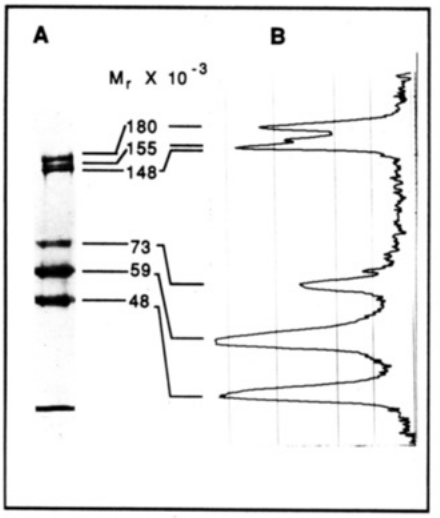

FIGURE 1: $NaDodSO_4$–PAGE of calf thymus DNA polymerase α–primase complex immunoaffinity purified with monoclonal antibody SJK287-38. Denaturing gel electrophoresis was performed as described under Materials and Methods. (A) Silver-stained gel of fraction 4 enzyme (5 μg of polymerase–primase). Standard protein markers were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (93 kDa), BSA (68 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). (B) A densitometric scan of the dried gel was performed as described under Materials and Methods. The stoichiometry of the subunit composition was calculated by considering the molecular mass of the subunits.

subunits are stainable to a similar extent. This assumption seems reasonable, since we observed a similar distribution of intensities with silver and Coomassie staining. A densitometric scan of a representative preparation is shown in Figure 1B. In this case, the relative intensities were 1:0.9:3.5:3.5 for the α-subunit cluster and the 73-, 59-, and 48-kDa polypeptide, respectively. The α-subunit cluster of this particular preparation consisted of 39% of a 180-, 16% of a 155-, and 45% of a 148-kDa polypeptide. In several preparations, minor bands (amounting to less than 10% of the totally stainable material) were detected with molecular weights of 210 000, 55 000, and 41 000. The relative amounts of the M_r 210 000 and 41 000 polypeptides were increased in preparations that were immunoaffinity purified directly from crude extracts (data not shown).

Physical Characterization of Immunoaffinity-Purified DNA Polymerase α. The sedimentation coefficient was determined by zonal centrifugation through a sucrose gradient. Pol-α and DNA primase activity sedimented together as a complex consisting of the α-subunit cluster and the three smaller subunits at 9 S in the presence of 0.5 M KCl (Figure 2). Part of the primase activity, displaying 30–50% of that measured for the entire complex, was separated from the complex. The separated primase activity cosedimented with polypeptides of M_r 59 000 and 48 000 (Figure 2B). This indicates that the smaller subunits, which are in a molar excess of the α-subunit(s), are rather loosely bound to the complex. A more detailed description of the properties of the free primase will be given elsewhere. Pol-α preparations obtained after extensive washing of the antibody column with 1 M NaCl showed a relative abundance of the α-subunits. When such preparations were subjected to zonal centrifugation, no separation of the primase was observed. In those cases, a second polymerase activity peak was detectable at about 6–7 S; this has also been reported by Chang et al. (1984).

The Stokes radius of the immunoaffinity-purified complex was determined in the presence of 0.5 M KCl by gel filtration on a calibrated Sepharose 6B column to be 6.0 nm (data not

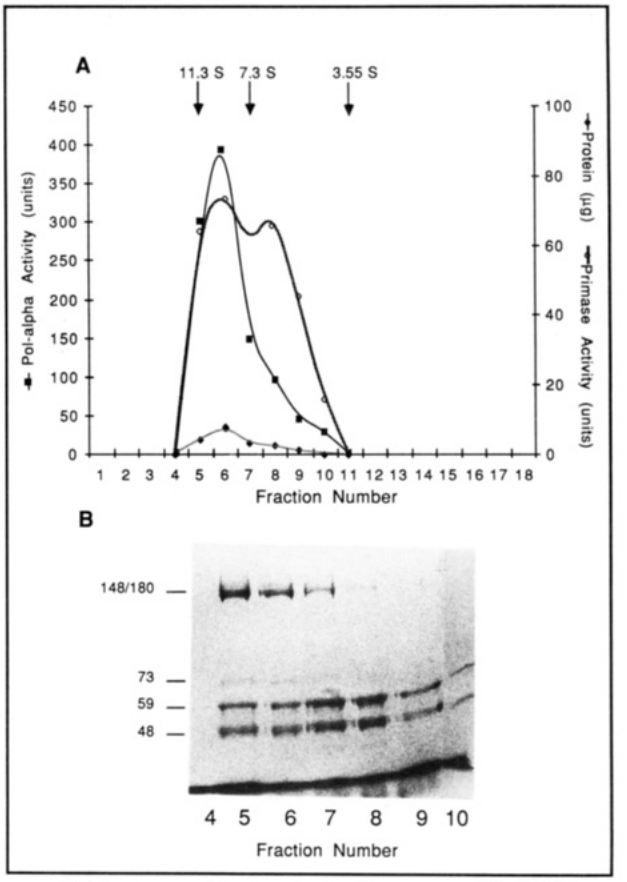

FIGURE 2: Sucrose gradient analysis of immunopurified DNA polymerase α–primase complex. 100 μg of fraction 4 enzyme was sedimented at 4 °C in a 5–20% (w/v) sucrose gradient at 40 000 rpm for 16 h in a Beckman SW56 rotor as described under Materials and Methods. At the end of centrifugation, fractions were collected from the bottom of the tube. (A) Aliquots of each fraction were assayed for protein (♦), polymerase (■), and primase activity (◊). Standard proteins, catalase (11.3 S), lactate dehydrogenase (7.3 S), and ovalbumin (3.6 S), were analyzed on a separate gradient. (B) 100 μL of each fraction (with the exception of fraction 6, where only 30 μL was taken to avoid an overloading of the gel) was precipitated with trichloroacetic acid and analyzed by denaturing gel electrophoresis.

shown). From this and the sedimentation coefficient of 9 S, one can calculate molecular weight of 335 000 for the native enzyme by following the method of Siegel and Monty (1966) and assuming a partial specific volume of 0.725 cm^3/g. A molecular weight of 335 000 is in resonable agreement with a 1:1:1:1 stoichiometry for the four subunits. The frictional ratio f/f_0 was estimated to be 1.31, which indicates a similar shape such as serum albumin or alcohol dehydrogenase, both of which display frictional ratios of 1.35 when measured under similar conditions (Siegel & Monty, 1966). In contrast, pol-α prepared by a different immunopurification protocol, but with the same monoclonal antibody, displays a Stokes radius of 9.6 nm and a frictional ratio of 1.96, indicating a high degree of molecular asymmetry (Wahl et al., 1984). The reason for this discrepancy is not yet understood.

Template-Primer Utilization of the Immunoaffinity-Purified DNA Polymerase α–Primase Complex. The immunopurified enzyme displays properties typical of pol-α. As shown in Table II, the immunoaffinity-purified enzyme is most active with activated DNA as a template-primer. With denatured DNA, it still exhibited 63% of its maximal activity, indicating a high potential for replicating long stretches of single-stranded DNA (see below). The enzyme is inhibited by *N*-ethylmaleimide and aphidicolin which proves that we

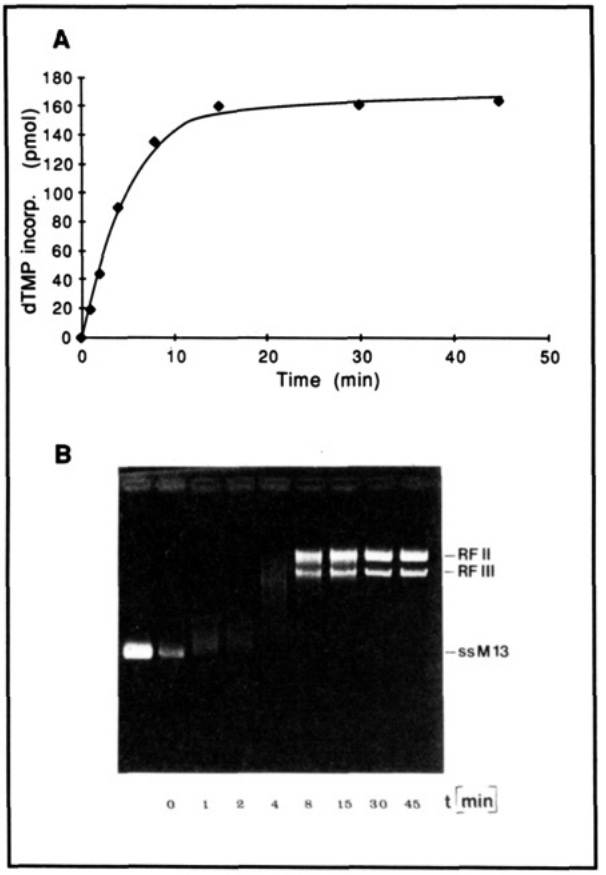

FIGURE 3: Replication of single-stranded M13 DNA by the immunopurified polymerase–primase. (A) M13mp8 (+) strand (50 μM) was replicated with 500 units/mL pol-α–primase in the presence of the four ribonucleoside triphosphates and the four dNTPs as described under Materials and Methods. At the times indicated, samples were withdrawn, and acid-precipitable radioactivity was determined as described under Materials and Methods. (B) Agarose gel electrophoresis of products of DNA synthesis on primase-primed M13 DNA. At the times indicated, aliquots were withdrawn and electrophoresed in a 1% neutral agarose gel for 6 h at 4 V/cm. Following staining with ethidium bromide, product bands were visualized with UV light.

Table II: Template-Primer Utilization of Immunoaffinity-Purified DNA Polymerase α from Calf Thymus[a]

reaction conditions	dAMP incorporated (% of maximum)
activated DNA	100
activated DNA + 30 μM aphidicolin	54 (47)
activated DNA + 10 mM NEM	0.3
activated DNA + 4 mM ATP	96
denatured DNA (calf thymus)	63
poly(dA)·(dT)$_{10}$	41 (14)
alternating poly(dA-dT)	21 (16)
M13 single-stranded DNA	0.1 (0.1)
M13 single-stranded DNA + 1 mM ATP	15
M13 single-stranded DNA + 1 mM ATP + 0.1 mM each of GTP, CTP, and UTP	25 (6.4)
standard assay, but 10 μM dNTPs	23.3 (2.4)
M13 single-stranded DNA, ori-primed	2.2 (0.7)
M13 double-stranded DNA (superhelical, RFI)	0.2
M13 double-stranded DNA (open circular, FRII)	0.1

[a] The experiments were performed as described under Materials and Methods with 2.4 units of fraction 4 polymerase α activity. DNA concentrations are 0.2 mg/mL for activated DNA, 50 μM (nucleotides) for M13 DNA, and 100 μM (nucleotides) for synthetic DNA. 100% incorporation represented 2.4 nmol of dAMP incorporated per hour with activated DNA under standard assay conditions, i.e., 0.1 mM dNTPs and 75 mM potassium acetate. Values in parantheses display relative incorporation rates (percent of maximum) for the conventionally purified pol-α.

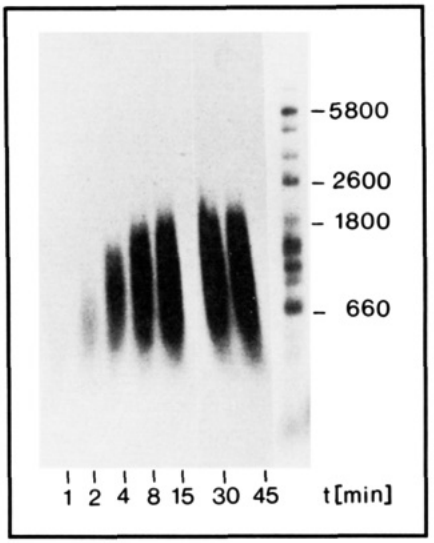

FIGURE 4: Alkaline agarose gel electrophoresis of the products of primase-primed replication on M13p8 (+) DNA. M13 DNA was replicated in the standard assay with 500 units/mL in the presence of ribonucleoside triphosphates, the four dNTPs, and [α-^{32}P]dATP. At the times indicated, aliquots were withdrawn and subjected to denaturing gel electrophoresis on a 1.5% alkaline agarose gel. Products were visualized by autoradiography. *Eco*RI-digested and 5′-labeled SPP1 DNA served as a lengths standard.

are dealing with an α species. Single-stranded M13 DNA was replicated at 0.1% of the rate of activated DNA. An analysis of the product lengths of this reaction by means of alkaline agarose gels revealed labeled products of more than 7000 nucleotides, indicating end addition at fold-back structures of linearized M13 DNA (data not shown). The addition of 1 mM ATP under otherwise identical conditions increased the reaction rate 150-fold. Moreover, in the presence of 1 mM ATP, and 0.1 mM each of GTP, UTP, and CTP, single-stranded M13 DNA was replicated at 25% of the rate of activated DNA (Table II). Neither superhelical nor nicked double-stranded M13 DNA was replicated to any appreciable extent. ATP, at concentrations of 0.1–5 mM, had no influence on the rate and extent of DNA synthesis on activated DNA or double-stranded DNA (Table II). On single-stranded DNA, however, ATP promotes priming (see above).

Immunoaffinity-Purified Pol-α Replicates Single-Stranded M13 DNA 3–5-Fold Faster Than Conventionally Purified Enzyme. As shown above, the immunoaffinity-purified DNA polymerase α replicates single-stranded M13 DNA very rapidly when rNTPs are present. In this reaction, dNMP incorporation was linear for 5–8 min. Thereafter, DNA synthesis levelled off, reaching 100% of its theoretical value (i.e., complete replication of M13 DNA) at about 15 min (Figure 3A). Figure 3B shows DNA replication as analyzed by neutral agarose gels. Considerable amounts of (nearly) completely replicated forms II and III DNA (RF III formation was mainly due to the presence of nicked M13 DNA in our stock solution) were formed within 8 min. Replication was completed within 30 min. From denaturing gels, the in vitro replicated DNA was found to have a distribution of product lengths from 600 to 2000 nucleotides (Figure 4). This is comparable to our earlier observations (Grosse & Krauss, 1985) and suggests (since the complete genome comprises 7229 nucleotides) that 3–10 primers are formed per M13 genome. For comparison, under identical conditions, 500 units/ml conventionally purified pol-α replicated the complete genome in 90–120 min (Grosse & Krauss, 1985). This rate was not increased when 10-fold higher concentrations of conventionally

Table III: Michaelis Constants for Deoxynucleoside Triphosphate Utilization, Inhibition by Aphidicolin, and Primer Utilization[a]

	K_m (K_i) (μM)	
	immunopurified pol-α	conventionally purified pol-α
dATP	0.8 ± 0.3	4.2
dGTP	0.6 ± 0.2	4.5
dCTP	0.7 ± 0.3	6.7
dTTP	0.9 ± 0.8	5.3
aphidicolin	0.6 ± 0.2	3.2
ori-primed M13 DNA	0.0035	0.040
unprimed M13 DNA	70	12

[a] K_ms for dNTP utilization and the K_i for aphidicolin inhibition were determined on activated DNA with three different preparations of immunoaffinity-purified DNA polymerase α–primase. The values are means ± SE of three determinations. Aphidicolin was found to be a competitive inhibitor to dCTP. The K_ms for primer utilization were determined on singly ori-RNA primed M13 DNA and are expressed in micromolar 3′OH primer ends. The K_is for unproductive binding to unprimed single-stranded DNA were determined by competing 40, 20, and 10 μM singly ori-RNA primed M13 DNA with various concentrations of unprimed M13 DNA. The K_is were calculated from a Dixon evaluation and are expressed in micromolar (nucleotide) single-stranded M13 DNA.

purified pol-α were used in the assay (data not shown).

Immunoaffinity-Purified Polymerase–Primase Displays Stronger Binding to Template-Primers and dNTPs Than the Conventionally Purified Enzyme. To find an explanation for the high rate of DNA synthesis on natural single-stranded DNA, we compared the enzymatic features of the conventionally purified polymerase with those of the immunoaffinity-purified enzyme. As shown in Table III, the K_m for binding to a unique RNA primer on M13 DNA was 3.5 nM for the immunoaffinity-purified polymerase and 40 nM for the conventionally purified enzyme. Moreover, the inhibition constants (K_i) for unproductive binding to single-stranded DNA of both polymerase preparations exhibited significant differences. The immunoaffinity-purified enzyme showed a K_i of 70 μM (nucleotide) M13 DNA whereas the conventionally purified enzyme is inhibited to 50% of its original activity by 12 μM (nucleotide) M13 DNA (Table III). Thus, the immunoaffinity-purified polymerase recognizes primers much easier in a vast excess of single-stranded DNA than the biochemically purified enzyme. Furthermore, the K_ms for the four dNTPs at 0.6–0.9 μM are also 5–10-fold lower than those exhibited by the conventionally purified enzyme (Table III). This holds for both template-primers, activated calf thymus DNA, and primase-primed single-stranded M13 DNA. DNA synthesis on primase-primed M13 DNA in the presence of only 10 μM each of the dNTPs proceeded at 93% of the rate when 100 μM (each) dNTPs were present (Table II). At 10 μM dNTPs, conventionally purified pol-α shows a rate of 40% of that observed at 100 μM dNTPs (Table II). The K_is for aphidicolin, which acts as a competitive inhibitor to dCTP (data not shown), were measured to be 3.2 and 0.6 μM for the conventionally purified and the immunoaffinity-purified pol-α preparations, respectively (Table III). An aphidicolin K_i of 0.22 μM was determined for the inhibition of replicative DNA synthesis in vivo (Pedrali-Noy & Spadari, 1979).

DNA Synthesis on Singly RNA-Primed M13 DNA Proceeds with a Maximal Rate That Approaches the in Vivo Rate of Fork Migration. The maximal rate of chain elongation on single-stranded M13 DNA was measured by performing a "run on" assay. For this purpose, DNA synthesis was initiated at a single preformed RNA primer at the M13 origin of replication (map position 5699; Geider et al., 1978). The progress of DNA synthesis up to the map position 3346, where

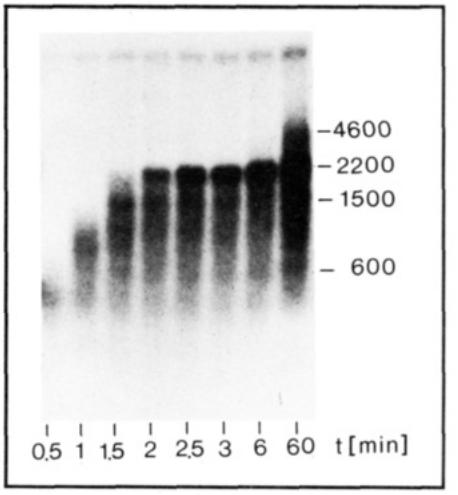

FIGURE 5: Singly RNA-primed replication of M13mp8 (+) DNA analyzed by denaturing agarose gel electrophoresis. Singly ori-RNA-primed M13 DNA (10 μM) was replicated in the standard assay with 500 units/mL pol-α–primase in the absence of ribonucleoside triphosphates (to avoid primase priming) but in the presence of dNTPs and [α-^{32}P]dATP. At the times indicated, aliquots were withdrawn, and the reaction was stopped by the addition of 0.1 volume of 1 M NaOH and subsequent heating to 65 °C. Products were electrophoresed on an alkaline 1.5% agarose gel and visualized by autoradiography. The numbers at the right indicate the lengths (in nucleotides) of *Hin*dIII-digested λ DNA and *Eco*RI-digested SPP1 DNA markers.

a stable hair-pin loop acts as a halting point for DNA polymerase α (Villani et al., 1981; Reckmann et al., 1985), was followed by product analysis on alkaline agarose gels. As shown in Figure 5, the stretch of 2350 nucleotides can be synthesized within 1.5–2 min. From this, one can calculate a maximal rate for chain elongation of about 26 nucleotides per second. This is close to the rate of fork migration inside the cell nucleus (Yurov, 1980). It should be noted, however, that the overall (initial) rate for DNA synthesis on singly primed M13 DNA comprises only 2.1% of the rate on activated DNA (Table II). Therefore, primer binding in a vast excess of single-stranded DNA is still rate limiting for the immunoaffinity-purified enzyme.

Immunoaffinity-Purified DNA Polymerase α Is More Active at High Salt Concentrations Than the Conventionally Purified Enzyme. Another important and novel characteristic of the immunoaffinity-purified DNA polymerase α is that it has considerable activity at rather high salt concentrations. As shown in Figure 6, the immunoaffinity-purified polymerase displayed an activity optimum at 120–180 mM potassium acetate (Figure 6A) and 90–150 mM KCl (Figure 6B) when activated DNA was used as a template-primer. In contrast, the conventionally purified enzyme was most active on activated DNA between 90–120 mM potassium acetate and 30–90 mM KCl. An even more pronounced salt effect was observed when the primase-primed M13 replication by both preparations was followed (Figure 6C,D). In general, the immunopurified enzyme was most active at about 90 mM potassium acetate (Figure 6C) or KCl (Figure 6D). By contrast, M13 replication with the biochemically purified enzyme was inhibited by any concentration of salt, independent of the anion used (Figure 6C,D). M13 replication is more sensitive to salts than DNA synthesis on activated DNA mainly because the primase is more salt sensitive (H.-P. Nasheuer and F. Grosse, unpublished experiments). However, it is noteworthy that the salt optimum of the immunopurified enzyme on self-initiated M13 DNA is similar to that observed for SV40 replication in nuclear extracts (Richter et al., 1980). As described by those authors,

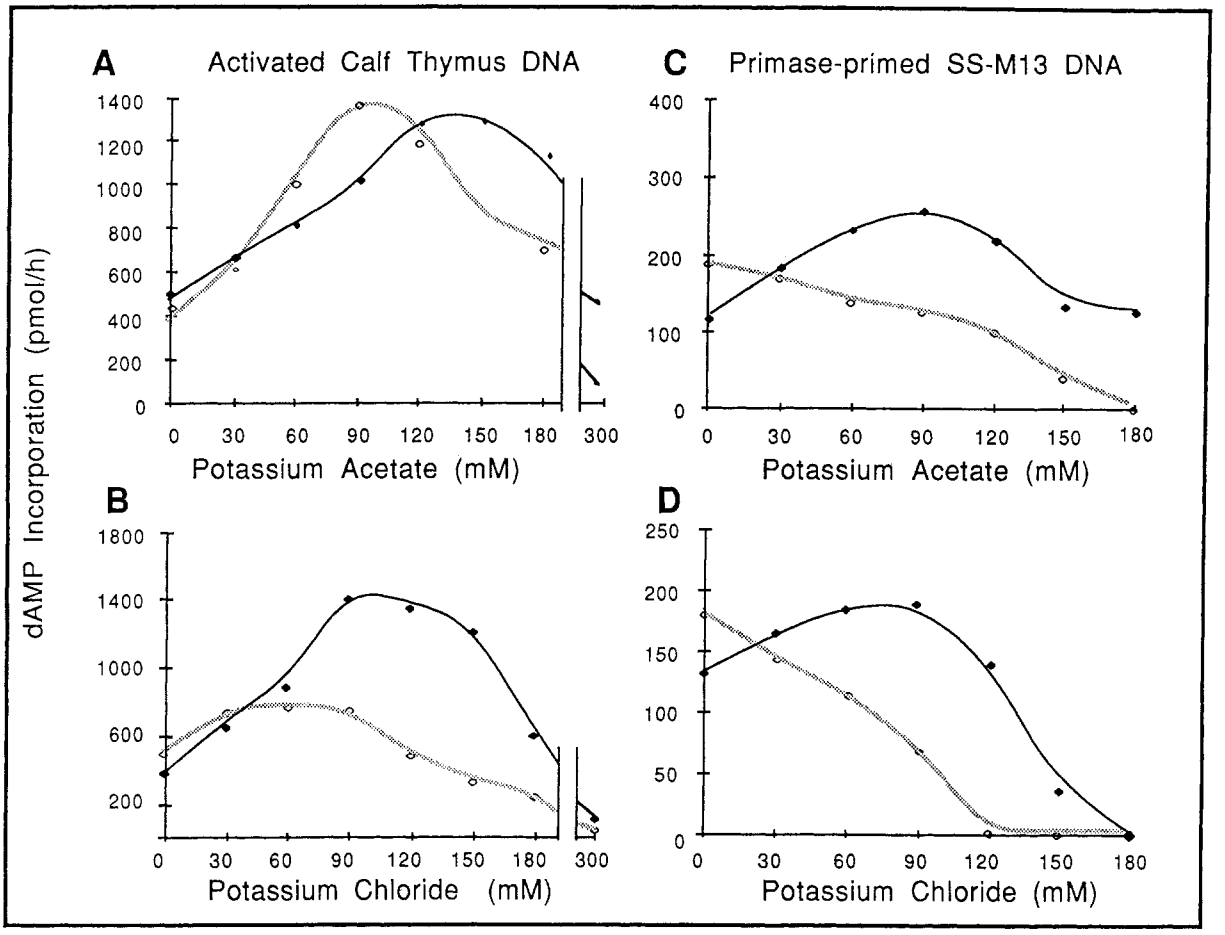

FIGURE 6: Salt dependence of immunoaffinity-purified and of conventionally purified polymerase–primase. DNA synthesis was measured in 20-μL samples of the standard polymerase assay with 2 units of the immunoaffinity-purified (♦) and the conventionally purified (◇) polymerase–primase on 0.2 mg/mL activated DNA (panels A and B) or 50 μM single-stranded M13 DNA in the presence of 1 mM ATP and 0.1 mM each of GTP, UTP, and CTP, in order to allow DNA priming. Salt concentrations for potassium acetate (A, B) and KCl (C, D) were as indicated on the abscissa. dAMP incorporation was determined from the linear part of the incorporation kinetics, usually after 3 and 6 min of incubation at 37 °C.

we found that the chloride anion is more inhibitory to pol-α than acetate. Nevertheless, with 120 mM KCl, the immunoaffinity-purified enzyme is nearly fully active in self-initiated M13 replication.

Discussion

Immunoaffinity chromatography has been successfully employed for the purification of pol-α from various species (Wahl et al., 1984; Chang et al., 1984; Wang et al., 1984; Masaki et al., 1984; Holmes et al., 1985, 1986; Wong et al., 1986). The subunit structures of these preparations are similar in that they all display a cluster of bands with molecular weights greater than 140000 and three subunits with molecular weights of 68000–78000, 55000–60000, and 41000–50000. The differences in molecular weights reported by different laboratories might result from the use of different molecular weight standards and therefore may not necessarily reflect different forms of the enzyme. The α-polymerase isolated by our procedure exhibits three polypeptides at 180, 155, and 148 kDa that are antigenetically related to each other [H.-P. Nasheuer and F. Grosse, unpublished experiments; see also Sauer and Lehman (1982), Karawya et al. (1984), Masaki et al. (1984), Holmes et al. (1985), and Wong et al. (1986)]. This cluster of bands most probably represent the DNA polymerizing activity. Three further subunits were observed at 73, 59, and 48 kDa which show no cross-reactivity with antibodies produced to the 148-kDa subunit. Of those, the 59- and 48-kDa polypeptides were shown to carry DNA primase activity, which is in accord with earlier results on biochemically purified pol-α from *Drosophila melanogaster* (Kaguni et al., 1983b) and later results with immunoaffinity-purified pol-α from calf thymus tissue (Holmes et al., 1986). Thus, in terms of enzyme structure, our preparation seems to be very similar to the preparations reported from other laboratories (Wahl et al., 1984; Chang et al., 1984; Wang et al., 1984; Wong et al., 1986). Despite these structural similarities, many key features of our immunoaffinity-purified enzyme strongly suggest that we are dealing with a more native enzyme than those described earlier by us and other authors. First, the in vitro replication of primase-initiated single-stranded M13 DNA is accomplished within 10–30 min. This is at least 3-fold faster than with the previously described conventionally purified enzyme (Grosse & Krauss, 1985) and seems to be also faster than another immunoaffinity-purified preparation from calf thymus tissue (Chang et al., 1984). It seems noteworthy to point out that the times required for complete replication of primase-primed single-stranded M13 (or ϕX174) DNA cannot be shortened by the addition of large amounts of pol-α–primase. RFII formation requires 90–120 min with the earlier purified enzyme and 10–30 min with the immunopurified enzyme, independent of whether 500 or 5000 units/mL enzyme is used in the assay mixture. Thus, besides the specific activity of pol-α on activated DNA, the time required for replication of natural single-stranded DNA represents an independent and in our opinion more stringent criterium on the quality of the corresponding pol-α preparation.

In order to find out what is responsible for the rather fast DNA replication on single-stranded M13 DNA, we studied several enzymatic parameters by directly comparing the immunoaffinity-purified DNA polymerase α with the biochemically purified enzyme. We found that the increased rate of single-stranded DNA replication is certainly not due to an increased priming frequency because both enzyme preparations initiate equally 3–10 times per single-stranded M13 DNA. The most striking differences were in primer binding and dNTP binding. The immunoaffinity-purified enzyme has a 10-fold higher affinity for a single RNA primer on M13 DNA than the corresponding biochemically purified enzyme. This is achieved by both a lower K_m for 3′OH binding and a higher K_i for unspecific binding to single-stranded DNA for the immunoaffinity-purified polymerase. Nevertheless, primer recognition in a vast excess of single-stranded DNA is still rate limiting for the immunoaffinity-purified enzyme, as demonstrated by the rather inefficient use of singly RNA primed M13 DNA in comparison to activated DNA. We also found 5–10-fold lower K_ms for binding of the four dNTPs, with both activated DNA and single-stranded M13 DNA. The submicromolar K_ms for dNTPs might result as a direct consequence of the stronger binding of the immunopurified enzyme to 3′OH primer ends, if one assumes that the ordered mechanism with the formation of the enzyme-primer complex first, and the subsequent binding of dNTPs second, as it was found for the human KB cell (Fisher & Korn, 1981; Wong et al., 1986) and the murine DNA pol-α (Detera et al., 1981), also holds for the calf thymus enzyme. The immunoaffinity-purified enzyme is relatively resistant to salt inhibition. With activated DNA as template-primer, maximal activity is observed at 150 mM potassium acetate and at 120 mM KCl. Self-initiated replication on M13 DNA is optimal at 90 mM monovalent cations, whereas the biochemically purified pol-α was inhibited at any concentration tested. Two other immunoaffinity-purified α-polymerases from calf thymus (Wahl et al., 1984) and human KB cells (Wang et al., 1984) are inhibited at higher salt concentrations and therefore seem to be more comparable to our conventionally purified enzyme.

From the data presented in this work, it is reasonable to assume that the DNA polymerase α, as purified by the given protocol, displays many properties that are required for proper in vivo function. It is not yet clear what might be responsible for the more native state of our enzyme. Lehman's group initially isolated a DNA pol-α from *Drosophila melanogaster* (Banks et al., 1979) that consisted of an α-subunit with a molecular weight of 148 000. Later, Kaguni et al. (1983) purified an "intact" form of the *Drosophila* enzyme, with an α-subunit of 182 kDa that displayed a 5-fold lower K_m for dTTP binding as compared to their earlier preparation. The intact enzyme was subsequently shown to replicate multiprimed M13 DNA with near in vivo rates and with a very high accuracy (Kaguni et al., 1984). Those authors concluded that proteolysis of the α-subunit leads to a functionally less native form of the polymerase–primase. A similar conclusion seems not to be valid for the calf thymus enzyme for the following reasons: First, immunoaffinity-purified preparations of pol-α that completely lack the 180-kDa subunit do not behave significantly different from preparations that contain a prominent 180-kDa subunit in all the assays employed. Second, in our hands, DNA polymerase activity seems to correlate with the 148-kDa polypeptide rather than with the 180-kDa polypeptide. It is not yet clear whether the elution conditions employed lead to a less active 180-kDa subunit, or whether the 180-kDa polypeptide represents a less active or even inactive preprotein. The latter assumption would be in accordance with a similar finding of Masaki et al. (1984).

Although the immunoaffinity-purified polymerase–primase exhibits many features of an intact and more native enzyme, it is most probably not a holoenzyme, comparable to the prokaryotic counterpart (McHenry & Kornberg, 1977). First, as it has been demonstrated with singly ori-RNA-primed M13 DNA, primer recognition is still rate limiting for DNA synthesis. Therefore, proteins that specifically increase primer binding over unspecific binding to single-stranded DNA might be necessary for a more efficient enzyme. Second, hairpin structures on natural single-stranded DNAs still serve as barriers to replication as has been found for the conventionally purified enzyme (Grosse & Krauss, 1984; Reckmann et al., 1985). Third, the primase of the immunopurified enzyme initiates about once per 1000 nucleotides, which is too low to explain the length of a eukaryotic Okazaki fragment, i.e., 100–200 nucleotides (Magnussen et al., 1973). Fourth, and most striking, the immunopurified enzyme exhibits a processivity of 18 (Hohn & Grosse, 1987), which again is not in agreement with the length of a eukaryotic Okazaki fragment. Therefore, further subunits or protein factors might be necessary that increase primer binding, the frequency of priming, and the processivity and that allow a faster replication through pausing sites. Efforts are under way to isolate such protein factors.

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Registry No. dATP, 1927-31-7; dGTP, 2564-35-4; dCTP, 2056-98-6; dTTP, 365-08-2; aphidicolin, 38966-21-1.

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Genetic Polymorphism of Human Cytochrome P-450 (*S*)-Mephenytoin 4-Hydroxylase. Studies with Human Autoantibodies Suggest a Functionally Altered Cytochrome P-450 Isozyme as Cause of the Genetic Deficiency†

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ABSTRACT: The metabolism of the anticonvulsant mephenytoin is subject to a genetic polymorphism. In 2-5% of Caucasians and 18-23% of Japanese subjects a specific cytochrome P-450 isozyme, P-450 meph, is functionally deficient or missing. We have accumulated evidence that autoimmune antibodies observed in sera of patients with tienilic acid induced hepatitis (anti-liver kidney microsome 2 or anti-LKM2 antibodies) specifically recognize the cytochrome P-450 involved in the mephenytoin hydroxylation polymorphism. This is demonstrated by immunoinhibition and immunoprecipitation of microsomal (*S*)-mephenytoin 4-hydroxylation activity and by the recognition by anti-LKM2 antibodies of a single protein band on immunoblots of human liver microsomes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis or isoelectric focusing. The cytochrome P-450 recognized by anti-LKM2 antibodies was immunopurified from microsomes derived from livers of extensive (EM) or poor metabolizers (PM) of (*S*)-mephenytoin. Comparison of the EM-type cytochrome P-450 to that isolated from PM livers revealed no difference in regard to immuno-cross-reactivity, molecular weight, isoelectric point, relative content in microsomes, two-dimensional tryptic peptide maps, one-dimensional peptide maps with three proteases, amino acid composition, and amino-terminal protein sequence. Finally, the same protein was precipitated from microsomes prepared from the liver biopsy of a subject phenotyped in vivo as a poor metabolizer of mephenytoin. These data strongly suggest that the mephenytoin hydroxylation deficiency is caused by a minor structural change leading to a functionally altered cytochrome P-450 isozyme.

Large interindividual variations in drug response in the population are frequently caused by differences in drug oxidation capacity by liver cytochrome P-450 isozymes. Several genetic polymorphisms in drug oxidation have been described (Mahgoub et al., 1977; Eichelbaum et al., 1979; Küpfer et al., 1979; Scott & Poffenbarger et al., 1979; Kleinbloesem et al., 1984). The hydroxylation polymorphism of the anticonvulsant drug mephenytoin is one of the best studied examples (Küpfer & Preisig, 1984; Inaba et al., 1984; Wedlund et al., 1984). It occurs in 2-5% of the white European and North American population and in 18-23% of Japanese subjects (Jurima et al.,

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