Synthesis of DNA by DNA Polymerase ε in Vitro*

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The isolation of DNA polymerase (Pol) ε from extracts of HeLa cells is described. The final fractions contained two major subunits of 210 and 50 kDa which co-sedimented with Polε activity, similar to those described previously (Svyatova, J., and Linn, S. (1989) J. Biol. Chem. 264, 2489–2497). The properties of the human Pol and the yeast Pol were compared. Both enzymes elongated singly primed single-stranded circular DNA templates. Yeast Polε required the presence of a DNA binding protein (SSB) whereas human Polα required the addition of SSB, Activator 1 and proliferating cell nuclear antigen (PCNA) for maximal activity. Both enzymes were totally unable to elongate primed DNA templates in the presence of salt; however, activity could be restored by the addition of Activator 1 and PCNA. Like Polα, Polε formed complexes with SSB-coated primed DNA templates in the presence of Activator 1 and PCNA which could be isolated by filtration through Bio-Gel A-5m columns. Unlike Polα, Polε bound to SSB-coated primed DNA in the absence of the auxiliary factors. In the presence of salt, Polε complexes were less stable than they were in the absence of salt.

In the in vitro simian virus 40 (SV40) T antigen-dependent synthesis of DNA containing the SV40 origin of replication, yeast Polα but not human Polε could substitute for yeast or human Polα in the generation of long DNA products. However, human Polε did increase slightly the length of DNA chains formed by the DNA polymerase α-primase complex in SV40 DNA synthesis. The bearing of this observation on the requirement for a PCNA-dependent DNA polymerase in the synthesis and maturation of Okazaki fragments is discussed. However, no unique role for human Polα in the in vitro SV40 DNA replication system was detected.

In eukaryotes, three distinct DNA polymerases have been implicated in chromosomal DNA replication. The first enzyme, DNA polymerase α (Pola)1 contains a catalytic subunit of 180 kDa associated with a 70-kDa subunit of unknown function. Polα can be isolated in a complex with DNA primases (subunits of 55 and 45 kDa), and the Polα-primase complex has been shown to be essential for the in vitro replication of SV40 DNA as well as chromosomal replication in vivo (Eki et al., 1986; Murakami et al., 1986; Stillman, 1989; Chalberg and Kelly, 1989). DNA primase catalyzes the initiation and synthesis of oligoribonucleotides which are elongated immediately by Polε. These DNA products (Okazaki fragments) arise from the lagging strand template. The second enzyme, DNA polymerase δ (Polδ) consists of a catalytic subunit of 125 kDa and another subunit of 50 kDa of unknown function (Lee et al., 1984; Goulia et al., 1990). In the presence of PCNA and a multimeric complex catalyzed Activator I (A1) or replication factor C (RF C), Polδ catalyzes the elongation of Okazaki fragments to long DNA chains, representing leading strand DNA synthesis in the SV40 replication reaction. Collectively, Polε, PCNA, and A1 have been called the Polδ holoenzyme. These two DNA polymerases, α and δ, are capable of supporting the in vitro replication of SV40 DNA (Pelich et al., 1987; Lee et al., 1989a; Weinberg et al., 1989; Tarasawa et al., 1990).

Recently, a third DNA polymerase, Polε, was shown to be essential in yeast (Morrison et al., 1990). yPolε was reported to be highly processive and consists of a catalytic subunit of 256 kDa, deduced from the gene sequence, which possibly is associated with 80-, 38-, and 35-kDa subunits of unknown function. Polε, prepared from HeLa cells and containing subunits of 215 and 50 kDa, was isolated initially as a PCNA-independent Polδ and has been implicated in DNA repair synthesis (Nishida et al., 1989). Recently, Burgers (1990) observed that the elongation of primed DNA templates by yeast Polα (Ypol) can be inhibited completely by relatively low concentrations of salt. This inhibitory effect, however, was reversed completely by the addition of PCNA and A1, the two auxiliary proteins essential for Polδ-catalyzed elongation of primed DNA templates.

In this report, the properties of yPolε and hPolε have been compared. hPolε and yPolε showed similar properties in catalyzing the elongation of primed DNA templates in the absence of salt. Both enzymes elongated (dT)12–18 efficiently, but differed in their requirements for the elongation of singly primed single-stranded circular (ssc)

1 The abbreviations used are: Polα, δ, ε, DNA polymerase α, δ, ε; SSB, single-stranded DNA binding protein; HSSB, human single-stranded DNA binding protein; YSSB, yeast single-stranded DNA binding protein; SV40, simian virus 40; T antigen, SV40 large tumor antigen; BSA, bovine serum albumin; PCNA, proliferating cell nuclear antigen; ss, single-stranded; ssc, single-stranded circular; A1, multimeric Activator I protein; kb, kilobase pair(s); h, y (as prefix), human, yeast; BuPhdGTP, N2-(p-n-butylphenyl)-2'-deoxyguanosine 5'-triphosphate; T4 g32, bacteriophage T4 gene product 32; T7 25, bacteriophage T7 gene product 25; DTT, dithiothreitol.

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DNAs, yPol required a SSB for this reaction, whereas hPol c required SSB, A1, and PCNA for maximal activity. However, in the presence of salt, both enzymes required SSB, PCNA, and A1 for the elongation of all primed DNA templates. Both Pol preparations formed a stable complex with SSB-coated primed DNA templates in the absence of ATP which could be isolated by gel filtration. The isolated complex catalyzed the elongation of primed DNA templates in the absence of salt. In the presence of salt, however, neither Pol preparation formed a stable complex with SSB-coated primed DNA templates even in the presence of A1 and PCNA. This suggests that the elongation reaction catalyzed by Pol may differ from that of Pol. The site(s) of Pol binding to primed DNA templates coated with SSB is unknown.

In the in vitro SV40 replication system, in conjunction with PCNA and A1, yPol supported the synthesis of long DNA chains in the presence of low concentrations of Pola-primase complex, HSSB, topoisomerase I, and T antigen. In contrast, hPol c did not support the synthesis of long DNA chains but did increase the size of Okazaki fragments synthesized by Pol. Our studies, however, have revealed no unique role for Pol in the in vitro SV40 replication reaction.

In vitro, the Pola-primase complex, SV40 T antigen, and HSSB are required specifically to carry out the initiation DNA chains in the SV40 DNA replication system (Matsumoto et al., 1990). In contrast, the elongation of these chains can be carried out by a variety of DNA polymerases (Tsurimoto et al., 1990; Matsumoto et al., 1990). Our studies indicate that yPol can participate in the elongation reaction almost as efficiently as yPol a and hPol a. However, if hPol c were to play an important role in the elongation reaction, another accessory factor(s) in addition to HSSB, A1, and PCNA would be essential.

MATERIALS AND METHODS

Preparation of DNAs and Proteins—Primed see M13 and φX174 DNAs were prepared by hybridization of the viral ssDNA with an oligonucleotide 30 nucleotides long, complementary to nucleotides 5127–5156 of φX174 and nucleotides 5614–5643 of M13 viral DNAs, as described previously (Lee et al., 1986). Preparations of poly(dA·oligo(dT))·T antigen (Phar-macia) and the SV40 origin containing DNAs, pSVLD (10 kb) and pSSB (10·1 kb) were prepared as described previously (Ishimi et al., 1988), and used as the template (2.5 μg) in place of poly(dA)·oligo(dT). In addition, various preparations used in the assay contained at least 1 ng of buffer A, each reaction contained 10 7·103 units each of Pola, dGTP, and dCTP in the presence of 10 μM BuPhdGTP, a potent inhibitor of Pola but not Pol (Nishida et al., 1988). All reactions were incubated for 30 min at 37 °C, and the amount of acid-insoluble DTP was determined. One unit of Pol supported the incorporation of 1 nmol of DTP under the conditions described directly in the poly(dA)·oligo(dT) assay.

Pol scored well in both assays, whereas Pola scored well in the activated DNA assay and poorly in the poly(dA)·oligo(dT) assay. For this reason, Pol was measured using the second assay. One unit of Pol supported the incorporation of 1 nmol of DTP after 30 min at 37 °C.

Cytosolic extracts of HeLa cells (derived from 90 liters), prepared as described previously (Wobbe et al., 1985), were adjusted to 65% saturation with solid ammonium sulfate (39.8 g/100 ml). After centrifugation, the precipitate was dissolved in 190 ml of buffer A (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 0.2 μg/ml leupeptin, 0.1 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride) containing 0.25 M NaCl and dialyzed against 4 liters of buffer A plus 0.05 M NaCl for 12 h. The dialyzed protein fraction (15.3 mg of protein/ml, 210 ml, 2394 units) was loaded onto a DEAE-Sepharose column (5 × 10 cm, 196 ml), equilibrated with 1.5 liters of buffer A containing 0.1 M NaCl and eluted at 4 liters of buffer A plus 0.025 M NaCl. Pol was eluted with a 1.68-liter gradient of 0.025–0.5 M NaCl in buffer A. The peak of Pol activity coeluted with Pol (0.2 M NaCl (3.5 mg of protein/ml, 151 ml, 1752 units) was diluted to 0.15 M NaCl with buffer A and loaded directly onto a phosphocellulose column (2.5 × 13 cm, 64 ml) preequilibrated with 1 liter of buffer A containing 0.15 M NaCl. The column was washed with 78 ml of buffer A plus 0.15 M NaCl and subsequently eluted with a 500-ml gradient of 0.15–0.5 M NaCl in buffer A. Fractions containing Pol activity, which coeluted with Pol at 0.27 M NaCl, were pooled and dialyzed against 1.5 liters of buffer A containing 0.1 M NaCl and 1.0 M phenylmethylsulfonyl fluoride (which was present in all subsequent purification steps described below) for 12 h. The dialyzed fraction (0.22 mg of protein/ml, 59 ml, 1534 units) was loaded onto a heparin-Sepharose column (1.5 × 3.3 cm, 5.8 ml), preequilibrated with 1 liter of buffer A plus 0.1 M KCl. The peak of Pol activity coeluted with Pol at 0.3 M KCl and was partially separated from Pol which eluted at 0.25 M KCl. The pooled Pol fraction (0.52 mg of protein/ml, 11 ml, 1804 units), containing a significant amount of contaminating Pola (2582 units in the absence of BuPhdGTP versus 1477 units in the presence of BuPhdGTP), was concentrated directly on hydroxylapatite at 300°C (1.0 × 2.6 cm, 2 ml) preequilibrated with 15 ml of buffer B (40 mM potassium phosphate buffer, pH 7.0, 2 mM DTT, 10% glycerol, 0.05% (v/v) Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 μg/ml antipain, and 0.1 μg/ml leupeptin). The column was washed with 4.5 ml of the same buffer and then eluted with a 21-ml gradient of 0.4–0.45 M potassium phosphate, pH 7.0, in buffer B. The Pol peak eluted at 0.12 M potassium phosphate buffer (overlapping with the Pola activity which eluted at 0.13 M potassium phosphate) and was pooled (0.34 mg of protein/ml, 6.5 ml, 1392 units) and dialyzed against 1 liter of buffer A containing 0.05 M NaCl. The dialyzed material was then loaded onto a small Q-Sepharose column (0.7 × 2.7 cm, 1 ml). After washing the column with 3.5 ml of buffer A plus 0.05 M NaCl, Pol activity was eluted with Pol at 0.24 M NaCl using an 11-ml gradient of 0.05–0.5 M NaCl in buffer A, and the active fractions were pooled (0.13 mg of protein/ml, 2.5 ml, 503 units). Using the polymerase assay with activated DNA, the Q-Sepharose fraction contained a total of 1132 units of polymerase activity that was reduced to 557 units when the assay was carried out in the presence of 10 μM BuPhdGTP. We have interpreted this observation to mean that 50% of the polymerase activity present in this fraction was due to the contaminating Pola activity, and the supernatant was used. The remaining beads were washed three times with 0.6 M of buffer A containing 0.2 M NaCl, and the supernatants were saved and assayed

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for polymerase activities. The presence of contaminating Polo activity in these SJK-132-20-treated samples was monitored by examining the influence of BuPhdGTP on the DNA polymerase activity by testing for DNA primase activity (which normally is associated with Polo but not with Pole) as described previously (Nishida et al., 1988; Lee et al., 1986). The antibody column removed more than 88% of the Polo activity, and the residual activity was removed by a second depletion step. In this step, 80% of the Pole activity was recovered with no change in the specific activity. Thus, the hPolc preparations increased from 1,600 to 23,000.

The hPole activity was purified further by glycerol gradient centrifugation, as described below (Fig. 1). In this step, the specific activity of the Q-Sepharose-antibody-treated preparation was increased from 1,600 to 23,000.

**RESULTS**

**Glycerol Gradient Centrifugation of hPole**—The Polo-depleted Q-Sepharose fraction of hPole was subjected to glycerol gradient centrifugation as described in Fig. 1A. A single peak of polymerase activity was detected with a shoulder of activity on the trailing side of the gradient.

**Fig. 1.** Analysis of hPole activity and its subunit structure by glycerol gradient centrifugation. The Q-Sepharose fraction of hPole (0.16 ml, 20.5 µg of protein depleted of Polo-primase activity) was layered on top of a 5-ml 20-40% glycerol gradient and centrifuged at 4 °C for 22 h at 190,000 × g. A, fractions collected from the bottom of the gradient were assayed for DNA polymerase activity with poly(dA) oligo(dT) as described under “Materials and Methods.” B, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the glycerol gradient fractions. The numbers at the top of the figure indicate the fraction analyzed. The molecular weight markers are indicated to the left of the figure, while lane A was the starting material used for the glycerol gradient step.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the glycerol gradient fractions (Fig. 1B) indicated that this step separated a substantial amount of the protein present in the IgG-treated Q-Sepharose fraction from the polymerase activity. The peak fractions of hPole (Fractions 9–13) possessed a specific activity of 23,000, representing a 13-fold enrichment of the hPole in this step with a recovery greater than 90%. Two protein bands (210 and 50 kDa) co-sedimented with hPole activity. Syvaoja and Linn (1989) reported that hPole preparations isolated from HeLa cells contained two major polypeptides of 215 and 55 kDa, similar in size to those present in our preparations (Fig. 1B). Thus, the size of hPole, as well as its activity with different DNA templates described here, were similar to those observed by Syvaoja and Linn (1989). In addition to the two bands which co-sedimented with hPole activity, a 65 kDa band was also detected. This band, however, was distributed across the entire gradient and may be due to an artifact.

The shoulder of activity detected in Fractions 16–18 has not been characterized further. However, when Fraction 16 was mixed with the Polo peak fraction (Fraction 12), the activity observed was additive.

**Elongation of Primed DNA Templates by yPole and hPole**—The requirements for yPole activity using a singly primed M13 ssDNA as the primer template are summarized in Table I. In the absence of salt, yPole required the addition of E. coli SSB (an amount that completely coated the ssDNA), for extensive DNA synthesis. The addition of the Polo auxiliary proteins A1 and PCNA had no effect on the SSB-dependent yPole activity. However, in the presence of 0.13 M NaCl, both PCNA and A1 were required, and E. coli SSB further stimulated deoxynucleotidyl incorporation 4-5-fold.

In the presence of (dA)₄₉₆6 oligo(dT)₁₂₋₁₈ and salt (potassium glutamate), both hPole and yPole required SSB, A1, and PCNA for activity (Table II). Similar results were obtained when NaCl was used in place of potassium glutamate (data not shown). In the absence of salt, the elongation of (dA)₄₉₆₆ oligo(dT)₁₂₋₁₈ by hPole and yPole were unaffected by the addition of SSB, A1, and PCNA. These results differed from those observed with yPole in the presence of singly primed M13 ssDNA, which required SSB. We suspect that the difference in requirements with the different primed DNA templates is due to the sequestration of yPole by single-stranded DNA which is much more extensive with singly primed M13 (or φX) ssDNA containing a 30-nucleotide duplex region than with (dA)₄₉₆₆ oligo(dT)₁₂₋₁₈ (nucleotide

**FIG. 1.** Analysis of hPole activity and its subunit structure by glycerol gradient centrifugation. The Q-Sepharose fraction of hPole (0.16 ml, 20.5 µg of protein depleted of Polo-primase activity) was layered on top of a 5-ml 20-40% glycerol gradient and centrifuged at 4 °C for 22 h at 190,000 × g. A, fractions collected from the bottom of the gradient were assayed for DNA polymerase activity with poly(dA) oligo(dT) as described under “Materials and Methods.” B, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the glycerol gradient fractions. The numbers at the top of the figure indicate the fraction analyzed. The molecular weight markers are indicated to the left of the figure, while lane A was the starting material used for the glycerol gradient step.

**Table I**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>dTMP incorporated pmol</th>
</tr>
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<tbody>
<tr>
<td>− NaCl, + NaCl</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>62.1</td>
</tr>
<tr>
<td>Omit SSB</td>
<td>1.8</td>
</tr>
<tr>
<td>Omit PCNA</td>
<td>57.1</td>
</tr>
<tr>
<td>Omit A1</td>
<td>55.4</td>
</tr>
<tr>
<td>Omit SSB and PCNA</td>
<td>1.3</td>
</tr>
<tr>
<td>Omit SSB and A1</td>
<td>1.2</td>
</tr>
<tr>
<td>Omit PCNA and A1</td>
<td>63.7</td>
</tr>
<tr>
<td>Omit PCNA, A1, and SSB</td>
<td>1.3</td>
</tr>
</tbody>
</table>

The complete reaction contained 0.2 µg of singly primed M13 ssDNA, 1.6 µg of E. coli SSB, 120 µM each dGTP, dCTP, and dATP and 50 µM [³H]dTTP (300 cpm/pmol), 2 mM ATP, 60 ng of A1, 100 ng of PCNA, 0.1 unit of yPole, and, where indicated, 0.13 M NaCl. Reactions were incubated for 30 min at 37 °C, and acid-insoluble material was then determined.
TABLE II

Properties of DNA Polymerase ε

<table>
<thead>
<tr>
<th>Additions</th>
<th>dTMP incorporated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>- Salt</td>
</tr>
<tr>
<td></td>
<td>hPole(pmol)</td>
</tr>
<tr>
<td>Complete</td>
<td>115</td>
</tr>
<tr>
<td>Omit PCNA</td>
<td>113</td>
</tr>
<tr>
<td>Omit HSSB</td>
<td>107</td>
</tr>
<tr>
<td>Omit PCNA and HSSB</td>
<td>126</td>
</tr>
<tr>
<td>Omit HSSB and PCNA</td>
<td>100</td>
</tr>
<tr>
<td>Omit HSSB and PCNA and A1</td>
<td>127</td>
</tr>
<tr>
<td>Omit HSSB, PCNA, and A1</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>128</td>
</tr>
</tbody>
</table>

Fig. 2. Influence of PCNA and A1 on the size of DNA products formed with yPolc activity in the absence or presence of salt. Reaction mixtures (30 μl) contained 40 mM Tris-HCl, pH 7.8, 1 mM DTT, BSA (100 μg/ml), 7 mM MgCl₂, 0.9 μg of E. coli SSB, 120 μM each dATP, dGTP, dTTP, dCTP, 33.3 μM [α-³²P]dCTP (12,000 cpm/pmol), 2 mM ATP, and either 0.12 μg of singly primed φX174 ssDNA (lanes 1–8) or M13 mp18 ssDNA (lanes 9–16). Where indicated, 60 ng of A1 and 0.1 μg of PCNA were added. Incubations were for 30 min at 37 °C. DNA was isolated and analyzed on 1.0% alkaline agarose gel electrophoresis for 14 h at 55 V.

The ratio of 20:1. In the presence of SSBs single strands are complexed, reducing the nonproductive binding of yPolc and increasing the binding of the enzyme to primer ends. In addition, hairpins in the ssDNA which might block polymerase movement can be reduced by the addition of SSB. The products formed by hPole included many chains of discrete sizes, suggesting that the secondary structure of the single-stranded regions of the template contained

![Fig. 3. Influence of A1, PCNA, and SSB on the amount and size of products formed by hPole with singly primed M13 ssDNA.](image)

![Additions](image)

Absence, only small products were formed (data not shown). In the presence of 0.1 M NaCl, however, yPolc utilized singly primed DNA templates poorly and extended chains only a short length (lanes 5 and 13) and A1 or PCNA alone did not affect this limited DNA synthesis (Fig. 2, lanes 6, 7, 14, and 15). However, the inhibitory salt effect was reversed totally by the combined action of SSB, A1, and PCNA (Fig. 2, lane 8 and 16) resulting in extensive elongation and yielding products which were similar in length to those formed in the absence of salt.

When the exposure of the autoradiogram described in Fig. 2 was increased 20-fold, the size of the products observed in lanes 13–15 averaged <500 nucleotides, and no full-length DNA was detected.

In contrast to yPolc, hPole elongated singly primed M13 ssDNA poorly in the absence of salt (Fig. 3, lane 1). The addition of E. coli SSB reduced the size of the products (Fig. 3, compare lanes 1 and 2), and the addition of either A1 or PCNA alone had no effect (Fig. 3, lanes 3 and 4). The combination of A1 and SSB (at levels that completely coated the single-stranded regions) did not increase DNA synthesis significantly but did increase the size of the DNA products³ (Fig. 3, lane 5, compared to lanes 2 and 3). The combination of A1, PCNA, and SSB did increase DNA synthesis by hPole (lane 8). In the presence of 0.1 M NaCl, DNA chains were elongated in the presence of A1 and PCNA, and this reaction was stimulated further by the addition of SSB (Fig. 3, lanes 15 and 16). The products formed by hPole included many chains of discrete sizes, suggesting that the secondary structure of the single-stranded regions of the template contained

³ We have detected an activity that has contaminated some A1 preparations which can stimulate the hPole activity in the absence of PCNA regardless of the presence of salt. This activity, in contrast to A1, was resistant to N-ethylmaleimide treatment. The preparations of A1 used here were relatively free of this activity, but it is possible that the increase in size of products formed in Fig. 3, lane 5, was due to a small amount of this poorly characterized factor. The stimulation of hPole activity observed in Fig. 3, lane 8, was sensitive to N-ethylmaleimide and dependent on addition of PCNA. These properties are those observed with A1.
barriers that created pause sites. With yPol, the accumulation of discrete sized products in the presence of SSB was not as pronounced (see Fig. 2). On longer exposure (10 times), the size of the products detected in Fig. 3, lanes 9-15, were <142 nucleotides.

Effects of Various SSBs on Pol Activity—Because E. coli SSB stimulated the elongation of singly primed DNA by yPol in the absence of salt, we examined other SSBs for this effect. As shown in Fig. 4A, E. coli and T7 (T7 gene product 2.5) SSB were more effective than YSSB and HSSB. T4 g32 was partially effective only at high concentrations. In the presence of salt and with (dA)_{1000} oligo(dT)_{12-18} as the primer template, in addition to A1 and PCNA, SSB was also essential for yPol activity (Fig. 4B). Under these conditions, YSSB and HSSB were more effective than E. coli SSB and T4 g32, and the reasons for these differences are unclear.

As described in Table II, in the absence of salt and in the presence of (dA)_{1000} oligo(dT)_{12-18}, neither hPol6 nor yPol required SSB for activity. The presence or absence of A1 and PCNA did not alter this observation (Fig. 5A) nor did relatively high concentrations of HSSB. However, in the presence of salt, with (dA)_{1000} oligo(dT)_{12-18} as the primer template, hPol6 was almost completely dependent on an SSB (also see Table II). The multisubunit HSSB and YSSB were more effective than the SSBs encoded by E. coli or T4 (Fig. 5B). These observations were similar to those made with yPol.

The Formation of Proteins Complexed with Primed DNA and Their Elongation by yPol—The hPol6 holoenzyme formed an ATP-dependent stable complex with SSB-coated primed DNA template which could be isolated by gel filtration. This procedure was used successfully to determine the individual steps and order of addition of proteins in the formation of the Pol6 holoenzyme complexed to primed DNA (Lee and Hurwitz, 1990). It was shown that in the absence of ATP, A1 first formed a complex with the SSB-coated primed DNA template which then formed a second complex with PCNA in the presence of ATP. The A1-PCNA-SSB-coated primed DNA complex then interacted with hPol6 to form a stable complex that supported DNA replication upon the addition of dNTPs.

Because yPol activity required A1 and PCNA in the presence of salt, the formation of stable complexes with yPol, A1, and PCNA was examined (Figs. 6 and 7). Reaction mixtures containing E. coli SSB-coated singly primed M13 ssDNA, A1, PCNA, yPol, and ATP were incubated for 2 min at 37 °C and then filtered through a Bio-Gel A-5m column at 4 °C. The excluded volume fractions were assayed for DNA synthesis in the presence of ATP and in the absence (Fig. 6A) or presence (Fig. 6B) of salt. The isolated complex, under both conditions, supported DNA synthesis and the amount of synthesis was hardly affected by the further addition of A1, PCNA, or yPol. These observations indicated that the isolated complex contained all of the components essential for DNA synthesis in the presence of salt (A1, PCNA, and yPol) in addition to SSB-coated singly primed DNA.

When the 2-min incubation at 37 °C was carried out in the absence of ATP (Fig. 6, C and D), the complex formed differed...
from that synthesized in the presence of ATP. As shown in
Fig. 6C, the complex supported DNA synthesis when assayed
in the presence of ATP and the absence of salt, and the amount
of activity was unaffected by supplementation of reaction mixtures
with A1, PCNA, or yPolc. However, when the
complex formed in the absence of ATP was assayed in the
presence of salt, the results differed (Fig. 6D). Reaction mixtures
supplemented with PCNA or with PCNA plus yPolc were active, but assay mixtures that were not supplemented further and those supplemented only with yPolc were inactive. These results indicated that both A1 and yPolc, but not PCNA, bound to the SSB-coated primed DNA template in the
absence of ATP. Assays carried out in the absence of salt
resulted in DNA synthesis because yPolc can elongate the
SSB-coated primed DNA template in the absence of the
auxiliary proteins (and was independent of ATP) (Fig. 6C).
However, the elongation reaction was carried out in the
presence of salt, both PCNA and A1 were essential. A1 and yPolc were bound to the SSB-coated primed DNA in the
absence of ATP, but the complex in the excluded volume fractions
required both PCNA and ATP for the binding of
PCNA to the A1-DNA complex. Thus, the major distinction
between yPolc and Polh (both yeast and human) is that yPolc
can bind to primed DNA in the absence of the accessory
proteins and the complex formed is stable to Bio-Gel A-5m
filtration. Polh does not bind to primed DNA coated with SSB
in the absence of the auxiliary proteins A1 and PCNA (Lee

We also examined the ability of yPolc to form a stable
complex with singly primed DNA coated with SSB in the
presence of salt. Reaction mixtures containing yPolc, A1,
ATP, 0.13 M NaCl, and SSB-coated M13 primed ssDNA
were incubated at 37 °C for 2 min in the absence (Fig. 7A)
and presence (Fig. 7B) of PCNA and filtered through a Bio-
Gel A-5m column previously equilibrated with buffer A containing
0.13 M NaCl. The fractions in the excluded volume
were collected and assayed for DNA synthesis in the absence
of salt. As shown in Fig. 7A, the excluded fractions from
reaction mixtures incubated without PCNA required supple-
mentation with both PCNA and yPolc for DNA synthesis.
When the reactions included PCNA in the incubation along
with A1, yPolc, SSB-coated primed DNA and ATP, the
complex in the excluded fractions still did not support DNA
synthesis (Fig. 7B). However, DNA synthesis occurred when
the excluded fractions were supplemented with yPolc. Supple-
mentation with PCNA and A1 alone did not allow DNA
synthesis. This suggests that while A1 and PCNA formed an
ATP-dependent stable complex with SSB-coated primed
DNA in the presence of salt, yPolc did not.

Formation of Complexes with hPolh—When hPolh was used in
place of yPolc, the complex which was formed was different.
Reactions containing HSSB-coated (dA)22712-oligo(dT)12-18, A1,
PCNA, hPolh, and ATP were incubated for 2 min at 37 °C,
gel-filtered, and the fractions in the excluded volume were
assayed for their ability to support DNA synthesis in the
absence (Fig. 8A) and presence (Fig. 8B) of salt. In both cases,
the excluded volume fractions supported DNA synthesis upon
addition of DTTP. This result indicated that A1, PCNA, and
hPolh were complexed to primed DNA which supported DNA
synthesis in the presence and absence of salt. However, in
both cases, the addition of hPolh increased the activity of the
excluded complex approximately 2-fold, suggesting that hPolh
is less tightly bound to the A1-PCNA-SSB-coated DNA com-
and PCNA with SSB-coated singly primed DNA in the presence of salt, A1 complexed poorly with the primer with poly(dA).

Replication in the presence of salt (Fig. 7B).

The excluded amount of hPolt, without A1 and PCNA, formed a stable complex with SSB-coated primed DNA template in the presence of salt (Fig. 7A) and absence (panels C and D) of 2 mM ATP. Mixtures were then gel-filtered and assayed for DNA synthesis as described in Fig. 6 in the absence of ATP, the excluded fractions were assayed for DNA synthesis as described in Fig. 6, in the presence of ATP, and absence (panels A and B) and absence (panels A and C) of 0.1 M NaCl. Each fraction was supplemented with protein factors (30 ng of A1, 0.1 µg of PCNA, and 0.1 unit of hPolt) as indicated: □, no addition; ○, A1; ■, PCNA; ●, hPolt, and △, hPolt and PCNA.

When the 2-min incubation at 37 °C was carried out in the absence of ATP, the excluded fractions supported poly(dT) synthesis in the absence of salt (Fig. 8C). However, when the excluded fractions were assayed in the presence of salt (Fig. 8D), the complex required the addition of PCNA or PCNA plus hPolt for DNA synthesis to occur. These results indicated that A1 and hPolt complexed to the primed DNA template in the absence of salt but PCNA did not. This finding with hPolt (Fig. 8, C and D) is similar to that observed with yPolt (Fig. 8, C and D).

However, when we examined the ability of hPolt to form a stable complex with SSB-coated primed DNA template in the presence of salt, the results differed from those obtained with yPolt. Reaction mixture containing hPolt, A1, ATP, SSB-coated poly(dA)-oligo(dT), and 0.13 M NaCl were incubated for 2 min at 37 °C in the presence (Fig. 9A) or absence (Fig. 9B) of PCNA. The reaction mixtures were gel-filtered through a column equilibrated with buffer A containing 0.13 M NaCl, and the excluded volume fractions were assayed for DNA synthesis in the absence of salt. As shown in Fig. 9A, the excluded fractions from reaction mixture incubated with PCNA supported DNA synthesis upon addition of dTMP, indicating that A1, PCNA, and hPolt formed a complex with primed DNA in the presence of salt. The excluded fraction from reaction mixtures incubated without PCNA, however, did not support DNA synthesis, as shown in Fig. 9B. The excluded fraction supplemented with PCNA and A1 supported 30% of the DNA synthesis seen in the reaction supplemented with PCNA, A1, and hPolt. This suggested that limiting amounts of hPolt, without A1 and PCNA, formed a complex with the HSSB-coated primed DNA. These results with poly(dA)-oligo(dT) coated with HSSB indicated that, in the presence of salt, A1 complexed poorly with the primer end. This contrasts with the stable complex formed by A1 and PCNA with SS-B-coated singly primed DNA in the presence of salt (Fig. 7B).

yPolt or Polα Can Substitute for hPolt in the SV40 DNA Replication in Vitro—The replication of DNA containing the SV40 origin is totally dependent on the presence of the SV40 T antigen, topoisomerase I (or II), HSSB, and the human Polα-prime complex (the polymerase system). In the absence of any one of these proteins, no DNA synthesis occurred. As shown in Fig. 10, in the presence of low levels of Polα-prime (0.02 unit of each), the replication of the 10-kb plasmid pSVLD containing the SV40 origin of replication yielded only short DNA chains (lane 1). The addition of hPolt, PCNA, and A1 resulted in the synthesis of long DNA products (lane 4) which required SV40 T antigen (lane 5). When yPolt was used in place of human Polα, long DNA products were also formed in the presence of A1 and PCNA, although their size was somewhat shorter than those obtained with hPolt (lane 8).

When yPolt was used in place of Polα, long DNA chains were formed and this synthesis required the presence of A1 and PCNA (Fig. 10, lanes 10–13). Interestingly, this effect was observed in the absence of salt, the conditions under which the elongation of primed DNA templates did not require PCNA and A1. The extent of synthesis observed with yPolt (lane 12) was approximately 50% of that observed with hPolt and yPolt.

When reactions with yPolt were carried out in the presence of 50 mM NaCl, the synthesis of small Okazaki DNA chains was reduced substantially due to the inhibition of the Polα-prime complex activity by salt. The omission of PCNA and the addition of A1 decreased the synthesis of Okazaki fragments even further (Fig. 10, compare lanes 14 and 15), reflect-

* The monomerization reaction carried out with 0.2 unit each of Polα and DNA primase was inhibited 24, 50, and 95% by 25, 50, and 100 mM NaCl, respectively, after 60 min at 37 °C. In the presence of lower levels of Polα and DNA primase, the inhibitory effects of salt are more pronounced.
dipolymerase replication system. of topoisomerase I, 0.4 described previously (Lee addition to dNTPs, rNTPs, and an ATP-regenerating system as elongation reaction mediated by Pola. In the presence of both either hPol6, yPolb, or yPolc were added. After incubation for 60 min products in the presence of salt was probably due to the gel electrophoresis. of SV40 origin-containing DNA (pSVLD) with hPolc was The DNA chains formed in this reaction were longer than those observed in the absence of salt. The synthesis of longer products in the presence of salt was probably due to the synthesis of a smaller number of primers which allowed the elongation reaction to occur unimpeded by the presence of intervening Okazaki fragments. Results similar to those observed in lane 16 were obtained previously when low levels of either Polα or DNA primase were used (0.01 unit of Pol α) in the dipolymerase system.2

The Replication of SV40 DNA with hPole—The replication of SV40 origin-containing DNA (pSVLD) with hPole was compared with the hPolβ, as shown in Fig. 11. The monopolymerase system (Fig. 11, lane 1) supported the synthesis of small DNA products (approximately 300 nucleotides long) due to the limiting concentration of Polα-primease used (0.05 unit of each). Long DNA chains were formed in the presence of hPolβ holoenzyme (lane 4, the dipolymerase system). The addition of hPole, A1, and PCNA to the monopolymerase system resulted in a slight increase in size of the DNA products (lane 6 compared to lane 2), suggesting that hPole did not function efficiently as a leading strand DNA polymerase. This effect was not due to the inhibition of the initiation of lagging strand DNA synthesis since the addition of Polβ to the reaction containing hPole resulted in extensive DNA synthesis (lane 7). Furthermore, the increase in the size of the products by Polβ or Polc was totally dependent on Polα-primease because, in its absence, virtually no synthesis occurred (lane 9). In the presence of 50 mM NaCl, virtually no products were detected in reactions lacking the Polβ holoenzyme. As mentioned above, the synthesis of small Okazaki fragments by the Polα-primease complex was inhibited by salt and the small number of lagging strand primers formed could be elongated by the Polβ holoenzyme resulting in the synthesis of long products with little or no detectable small fragments (Fig. 11, lanes 13 and 16). To evaluate more carefully the size of DNA products formed in reactions containing the monopolymerase system alone and the monopolymerase system supplemented with hPole, electrophoresis was carried through an alkaline gel containing 2% agarose (Fig. 12A) rather than a 1.2% agarose gel. The size of chains formed in the reactions containing the hPole, A1, and PCNA were longer than those formed without the accessory proteins (Fig. 12A, compare lane 4 with lanes 2 and 3). Thus hPole can extend Okazaki fragments formed by the Polα-primease complex to a limited extent.

Further indications that hPole can elongate Okazaki fragments generated by the Polα-primease complex were obtained by exploiting the inhibition of the monopolymerase reaction with poly(ADP-ribose)polymerase (Fig. 12B). We previously demonstrated that relatively high concentrations of Polα-primease (in the presence of T antigen, HSSB, and topoisomerase I) supports the synthesis of leading and lagging strands in the SV40 replication system (Ishimi et al., 1988). In the presence of poly(ADP-ribose)polymerase, leading strand synthesis was blocked and only short chains arising from lagging strands were formed (Lee et al., 1989a; Eki and Hurwitz, 1991). Similarly, high concentrations of A1 and PCNA also can lead to the cessation of leading strand formation in the monopolymerase reaction, although this block is dependent on the amount of Polα-prime complex. Monopolymerase reactions, inhibited with A1 and PCNA can be reversed by the addition of Polβ. Thus, the block of leading
Because hPole can elongate chains in the presence of A1 and PCNA, we examined whether hPole could substitute for Pol6 and work with A1 and PCNA to reverse the inhibitory effects of poly(ADP-ribose)polymerase (Fig. 12B). As shown in lanes 1 and 2, the monopolymerase system supported the production of long and short DNA chains and at the high level of Pola-primase complex added (0.2 unit each), A1 and PCNA only slightly reduced leading strand synthesis. The addition of poly(ADP-ribose)polymerase blocked the synthesis of long chains, and the addition of A1 and PCNA with poly(ADP-ribose)polymerase further inhibited synthesis and resulted in yet smaller chains (lane 4). When the dipolymerase system was used (lanes 5–8), the reversal of the poly(ADP-ribose)polymerase-mediated blocking of leading strand synthesis was observed only with the combined presence of Pol6, A1, and PCNA. When hPole (lanes 10–13) was used in place of Pol6, a partial reversal of the poly(ADP-ribose)polymerase inhibition was observed only in reactions containing both A1 and PCNA. However, the length of the DNA chains formed with hPole were distinctly shorter than those observed with Pol6, and the extent of DNA synthesis was also lower. It should be noted that hPole stimulated nucleotide incorporation (compare lanes 4 and 13).

**DISCUSSION**

hPole initially was isolated as a soluble factor required for DNA repair synthesis in permeabilized UV-irradiated fibroblast cells. It was classified originally as a PCNA-independent Pol based on its associated 3'-5' exonuclease activity, resistance to BuPhDGT/P (a known potent inhibitor of Pol6), its sensitivity to aphidicolin, and its lack of responsiveness to PCNA (Nishida et al., 1988). In yeast, the Pole (PolII) gene was cloned, sequenced, and shown to be a third essential DNA polymerase in addition to Pole (yeast PolI) and Pol6 (yeast PolIII) for chromosomal DNA replication (Morrison et al., 1990; Burgers et al., 1990).

A summary of the properties of yPole and hPole and the influence of salt on their ability to support elongation of primed templates are presented in Table III. In the absence of salt, the elongation of (dA)400–oligo(dT)12–18 by both enzymes required no additional factors. In the presence of salt, extension of the synthetic primer template required ATP, SS8, A1, and PCNA. The three-subunit YSSB or HSSB were most effective in supporting this reaction. The requirements for elongation of a singly primed ssDNA (M13 or φX174) containing a 30-nucleotide primer differed from those observed with poly(dA)400–oligo(dT)12–18. In this case, in the absence of salt, yPole required an SSB and elongated such templates extensively. *E. coli* SSB and T7 SSB were more effective than the YSSB or HSSB for this activity. The hPole worked poorly with singly primed templates, and in the absence of salt required SSB, A1, and PCNA for activity. In this case, *E. coli* SSB was more effective than the multiple subunit SSBs. The requirement for SSB in the absence of salt probably reflects the nonproductive binding of Pol6 to single-stranded DNA and removal of hairpin structures by SSB. With (dA)400–oligo(dT)12–18, at a nucleotide ratio of 20:1, the

![Fig. 12. A, effect of hPole on the SV40 monopolymerase replication system. Reaction conditions were the same as those described in Fig. 11. Where indicated, 0.6 μg of SV40 T antigen (Tag), 0.02 unit each of Pola-primase (Pole-Pri) complex, 60 ng of A1, 0.1 μg of PCNA, and, where indicated, 0.1 unit of Pol6 or 0.15 unit of hPole were added. After 60 min at 37 °C, the reaction products were analyzed by electrophoresis through a 2% alkaline agarose gel for 20 h at 60 V. B, the influence of poly(ADP-ribose)polymerase (PARP) on the monopolymerase system; comparison of the reversal of inhibition by Pol6 and hPole. Reaction mixture (40 μl) contained 0.23 μg of strand synthesis by poly(ADP-ribose)polymerase can be prevented by the supplementation of the reaction with A1, PCNA, and Pol6. The influence of poly(ADP-ribose)polymerase (PARP) on the monopolymerase system; comparison of the reversal of inhibition by Pol6 and hPole. Reaction mixture (40 μl) contained 0.23 μg of

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**Table III. Influence of Salt on the Monopolymerase System**

<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>DNA Polymerase</th>
<th>SSB</th>
<th>A1</th>
<th>PCNA</th>
<th>PARP</th>
<th>dCMP incorp (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No salt</td>
<td>Pole</td>
<td></td>
<td></td>
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<td></td>
<td>6.8 + + + + + + +</td>
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<tr>
<td></td>
<td>hPole</td>
<td></td>
<td></td>
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<td></td>
<td>6.8 + + + + + + +</td>
</tr>
<tr>
<td>Salt</td>
<td>Pole</td>
<td></td>
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<td></td>
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<td>6.8 + + + + + + +</td>
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<td></td>
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<td>6.8 + + + + + + +</td>
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*pSV01AP* (2.8 kb), 0.8 μg of HSSB, 0.2 unit of Pola-primase complex, 10° unit of topoisomerase I. Where indicated, 0.9 μg of SV40 T antigen, 0.1 μg of PCNA, 60 ng of A1, 1.2 μg of poly(ADP-ribose)polymerase, 0.2 unit of hPole, and 0.2 unit of hPole. After incubation for 60 min at 37 °C, DNA was isolated and analyzed by electrophoresis through a 1.2% alkaline agarose gel.
number of primer ends present are much larger than in singly primed M13 or αX sscDNA, and the single-stranded regions are much more extensive with M13 or αX DNA than with the synthetic DNA. However, hPol6 differed from yPol6 because the higher eukaryotic enzyme required the auxiliary proteins for activity suggesting that it is less able to find low levels of primer ends than the yeast enzyme. For this purpose, the auxiliary proteins probably facilitate this selective binding.

In the presence of salt, both yPol6 and hPol6 required the auxiliary proteins as well as an SSB. In contrast to the findings with (dA)4500-oligo(dT)12-18, singly primed templates were more efficiently elongated in the presence of E. coli SSB than with YSSB or HSSB. The reasons for this discrepancy are not clear, and further studies of these different SSBs may shed more light on this difference.

Both yPol6 and hPol6 behaved differently than yPol6 and hPol6 in forming complexes with primed DNA templates. With the singly primed DNA template, both Pol6 preparations can bind to SSB-coated DNA in the absence of salt while Pol6 did not (Lee and Hurwitz, 1990). In the presence of salt, neither Pol6 preparation bound to primed DNA. Under all conditions used, the order of activation of primer ends by auxiliary proteins indicated that A1 binds first followed by the ATP-dependent binding of PCNA. The last component added to the complex is the polymerase and this was true for Pol6 as well as Pol6. In the presence of (dA)4500-oligo(dT)12-18, the stability of A1 at the 3' ends was somewhat salt-sensitive (Fig. 9B), in contrast to the binding of A1 to the 3' end of a singly primed DNA. Whether this is due to some sequence-dependent stability, the influence of E. coli SSB versus HSSB, or reflects the effects of the presence of many more primer ends (present in the synthetic primed template) is not clear.

The functional properties of hPol6 described above differed from hPol6 whose activity is totally dependent on Pol6, PCNA, and SSB in the absence or presence of salt (Table III). Both yPol6 and hPol6 form stable complexes with A1-PCNA-primed DNA in the presence of ATP, and they can be isolated by gel filtration even in the presence of salt. It is possible that in the presence of salt, hPol6 and yPol6 form a transient complex with the A1-PCNA-primed DNA complex which is not stable to the gel filtration step. These findings suggest that during elongation reactions, the processivities of Pol6 and Pol6 may differ. As shown by Burgers (1991), this appears to be the case for the yeast enzymes.

It has been shown that the in vitro synthesis of leading strand DNA in the SV40 replication system is not specific and can be carried out using the Pol6 holoenzyme, high concentrations of Pol6, E. coli PolIII holoenzyme, T4 holoenzyme, T7 polymerase, and other DNA polymerases (Tsuriel and Kaufmann, 1990). This list now includes both yPol6 and yPol6 (Fig. 11). Interestingly, both yPol6 and yPol6 required A1 and PCNA for the synthesis of long DNA chains even in the absence of salt. In contrast, hPol6 did not support the synthesis of long DNA chains even in the presence of A1 and PCNA (Fig. 11). However, hPol6 did increase the size of Okazaki fragments synthesized by low levels of Pol6 in the presence of A1 and PCNA (Fig. 12A).

Nethanel and Kaufmann (1990) have reported that small lagging strand DNA fragments (<40 nucleotides in length) synthesized with crude extracts in the in vitro SV40 DNA replication system were inhibited selectively by BuPhdGTP, while the synthesis of longer Okazaki fragments (up to 250 nucleotides) from the lagging strand template in the SV40 system were less affected by this inhibitor. Bullock et al. (1991) also observed that the size of DNA fragments arising from the lagging strand template were increased from an average length of 300 nucleotides to 40–60 nucleotides by the addition of neutralizing antibodies against PCNA during pulse-chase experiments with HeLa cytosolic extracts. This effect was reversed by the addition of excess purified PCNA. These observations suggest that a PCNA-dependent polymerase may be involved in the maturation of small DNA fragments arising from the lagging strands which were initiated and only partially elongated by the Pol6-primase complex. Because the A1-PCNA complex at 3'-OH ends of primers can be utilized by either Pol6 or Pol6, it will be of interest to determine which PCNA-dependent polymerase contributes to the elongation of the lagging strand products initiated by the Pol6-primase complex.

During the purification of hPol6, we encountered considerable difficulty in separating Pol6 from Pol6. It is possible that this was due to an interaction between these polymerases. If such a complex were to exist, this could position Pol6 with or near Pol6 which could facilitate the combined action of these polymerases in lagging strand synthesis. Based on the studies presented here, both hPol6 and yPol6, in the presence of A1 and PCNA, synthesis long DNA products in the SV40 system more efficiently than either the yPol6 or hPol6. Whether Pol6 plays any role in SV40 DNA replication is not clear at present.

It will be necessary to develop neutralizing antibodies that differentiate Polε and Polδ to clarify which PCNA-dependent polymerase plays a role in lagging strand synthesis.

Recently, HSSB was shown to be involved directly in excision-repair DNA synthesis (Coverley et al., 1991). Circular duplex M13 DNA containing one 2-(acetylamino)fluorene adduct per circle at a specific guanosine residue after UV irradiation was repaired effectively when incubated with HeLa cell-free extracts. This repair synthesis was inhibited substantially by anti-HSSB monoclonal antibodies, and the addition of highly purified HSSB reversed this effect. Because mulisubunit SSBs (HSSB and YSSB) showed some specificity in stimulating hPolε activity on a primed DNA (Fig. 6), it is possible that hPolε functions as a repair polymerase. This function would be in keeping with the original assay used for its isolation (Nishida et al., 1988). The utilization of the same auxiliary proteins (A1 and PCNA) for repair and replication has a precedence. In E. coli, PolII and PolIII, enzymes involved in repair and replication, respectively, are both activated by the γ-δ complex and β subunit (Wickner and Hurwitz, 1974; Hughes et al., 1991).

At relatively high ionic strength, nonspecific binding of proteins to a variety of macromolecules usually is reduced. Here, we have shown that the role of the polymerase auxiliary proteins A1 and PCNA can overcome the inhibitory effects of salt on Polε. It is likely that in the absence of salt this enzyme finds available primer ends through its ability to bind and dissociate from single-stranded regions. Single-stranded DNA coated with SSB and salt reduce such nonspecific binding reactions. Primer ends containing A1 and PCNA are specific sites at which both Polα and Polε bind. Thus, high ionic strength can be used to visualize more specific protein-macromolecular interactions.

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