Expression of Herpes Simplex Virus Type 1 DNA Polymerase Gene by In Vitro Translation and Effects of Gene Deletions on Activity

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A cloned herpes simplex virus type 1 DNA polymerase gene which is biologically functional was inserted into SP6 and T7 promoter-containing vectors for in vitro transcription-translation. pol-specific RNA synthesized in vitro will direct the synthesis of a 140-kilodalton polypeptide in rabbit reticulocyte lysates. RNAs prepared from pol templates linearized at internal restriction sites specified deleted polypeptides with sizes consistent with colinearity of the pol gene and the 140-kilodalton primary translation product. The in vitro translated pol gene product was enzymatically active, with salt resistance and sensitivity to acyclovir triphosphate, similar to the enzyme activity in crude extracts of herpes simplex virus type 1-infected Vero cells. An in-frame deletion of 78 residues (amino acids residues 881 to 959) was introduced into the expression vectors to investigate the function of a region of the polypeptide (amino acids residues 881 to 895) which is conserved in nine other DNA polymerases. In a complementation assay, this mutation abolished biological activity as well as the enzymatic activity of the in vitro translated product. A BAL31 mutation deleting the upstream open reading frame of pol had no effect on biological activity in a complementation assay but was found to increase the efficiency of in vitro translation of pol RNA. Two amino-terminal deletions of 27 and 67 residues were found to greatly enhance the enzymatic activity of the in vitro translated product, while all carboxy-terminal deletions examined (the smallest being 164 residues) abolished in vitro enzymatic activity. Expression of the 67-residue amino-terminal deleted pol gene in Escherichia coli, using a bacteriophage T7-based system, resulted in accumulation of large amounts of an insoluble fusion protein. An antiserum prepared against this fusion protein precipitated the 140-kilodalton DNA polymerase from herpes simplex virus type 1-infected cell lysates.

Herpes simplex virus (HSV) DNA polymerase is an important model for the study of viral DNA replication and the mechanisms of antiviral drugs. This enzyme is essential for viral DNA replication and has been characterized genetically through the use of temperature-sensitive and drug resistance mutations which can be shown to affect the in vitro properties of the partially purified enzyme (2, 3, 22, 45). Several enzymatic activities of the polymerase have been characterized: (i) the DNA chain elongation activity, which is salt stimulated; (ii) a 3'-5' exonuclease activity; (iii) deoxynucleoside triphosphate-binding site; (iv) a pyrophosphate-binding site; and (v) a DNA-binding site (10, 11, 18, 42). The localization of these activities within the 140-kilodalton (kDa) protein molecule is not apparent, and, unlike the procaryotic DNA polymerases such as Escherichia coli polymerase I or T4 DNA polymerase, no clear evidence for functional domains exists for HSV polymerase. However, mapping and sequencing of drug resistance mutations reveal a clustering of these mutations in the carboxy-terminal half of the polymerase-coding sequence (5, 8, 9, 15, 22).

The HSV polymerase appears to be homologous with three procaryotic and five eucaryotic viral DNA polymerases (1, 13, 15, 20, 22, 30, 43, 48). At least three regions of strongly conserved amino acid sequences can be found in this class of DNA polymerases, but no functional significance of this observation is yet apparent. The HSV polymerase gene (pol) has been cloned, expressed in COS-1 cells from the simian virus 40 early promoter, and shown to specify a functional product by complementation of a temperature-sensitive DNA polymerase mutant (12). The expression of a functional polymerase gene product should help provide a detailed understanding of the mechanism of viral DNA replication and its inhibition.

In this report, we describe the expression of the HSV polymerase gene by in vitro transcription-translation and the effects of several deletion mutations on the enzymatic activity obtained by in vitro translation. To investigate the functional significance of evolutionally conserved amino acid sequences and the possible existence of functional domains within the HSV DNA polymerase, a full-length pol gene has been inserted into a T7 transcription vector, pol mRNA synthesized in vitro by T7 RNA polymerase can be translated in rabbit reticulocyte lysates to yield enzymatically functional HSV DNA polymerase. With this system, the effect of gene deletions on enzyme activity can be studied.

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MATERIALS AND METHODS

Cells and virus. Vero cells were used for plaque assays and stock production of the wild-type HSV type 1 (HSV-1; strain 17), HSV-1 strain KOS, and the temperature-sensitive DNA polymerase mutant tsC7 (2, 45). COS-1 cells were grown in modified Dulbecco minimum essential medium with 5% fetal calf serum at 37°C in 10% CO₂ (17). For complementation studies, COS-1 cells were transfected with carrier and plasmid DNAs by the DEAE-dextran technique (46). Some 48 h later, the cells were infected with tsC7 at a multiplicity of 5. Virus outputs were determined as described previously by titration on Vero cell monolayers at 33 and 39°C; the
complementation index was determined as described previously (12).

Plasmid constructions and bacterial strains. Plasmid constructions were carried out by standard methods in E. coli HB101, and M-13 bacteriophage DNA templates were prepared from strain JM101 (32, 49). For expression of the pol fusion protein in E. coli, strain HMS174 containing plasmid pGP1-2, maintained at 30°C on kanamycin, was used (47).

For construction of the in-frame deletion 701, a 3.5-kilobase (kb) PstI-SacI fragment of pD700 (pD7) (12) was inserted into mp18 to yield mpPOL3. The 0.8-kb FspI-SacI and 148-base-pair FspI-PsiI mpMPOL3 subfragments were inserted into mp18 to yield mpPOL3Δ. After verification by sequencing, the 0.7-kb MsIII-NotI fragment of mpPOL3 was inserted into a complementary 8.4-kb MsIII-NotI fragment of pD700 to yield pD701. For construction of the BAL31 deletion 702, plasmid pWM121 (3) was linearized at BamHI, treated with BAL31, ligated with HindIII octamer linkers, circularized, and transformed into E. coli HB101. Plasmids were screened by restriction mapping for the presence of an appropriate deletion between HindIII and BglII sites and then sequenced with double-stranded plasmid DNA (6). A 0.7-kb HindIII-BglII fragment of pWM121 ΔBAL31 was inserted into an 8.6-kb HindIII-BglII fragment of pD700 to yield pD702. Templates for in vitro translation were constructed by obtaining HindIII-SspI fragments of pD700, pD701, and pD702 and inserting them between the HindIII and HindIII sites in the polylinker cloning sites of pGEM1 (downstream from the SP6 promoter) and pGEM2 (downstream from the T7 promoter). The pGEM vectors were obtained from Promega BioTech, and pT7-7 was the gift of Stanley Tabor of Harvard Medical School. For construction of the T7 expression vector T7-7-1, a SnaBI-SspI fragment of pD702 was purified and ligated with a BamHI linear of T7-7 which had been inactivated with Klenow enzyme (New England BioLabs, Inc.). For construction of pT7-7-2, an EcoRI linear of pT7-7 was inactivated with Klenow enzyme, further treated with Clal, and then gel purified. The modified pT7-7 linear was ligated with a 583-base-pair Nael-BglII fragment from pWM121 and a gel-purified 5.6-kb Clal-BglII fragment of pT7-7-1.

DNA polymerase assays. Assays were carried out in 100-μl volumes containing a mixture of 10 μg of activated calf thymus DNA, 100 mM ammonium sulfate, 50 mM Tris hydrochloride (pH 8.0), 50 μg of bovine serum albumin, 0.5 mM dithiothreitol, 7.5 mM MgCl2, 5 μM each dATP, dGTP, and dTTP, and 2.5 μCi of [α-35S]methionine (3,000 Ci/mmol) (22). Programmed reticulocyte lysate, or another enzyme source, was added, and incubation was at 37°C for 20 min unless indicated. The reaction was stopped by addition of 0.8 μl of 10% trichloroacetic acid, set on ice for 10 min, and then spotted on Whatman GF glass filters on a membrane filter manifold (Millipore Corp.). Filters were washed three times with 2.0 ml of 10% trichloroacetic acid and once with 2.0 ml of absolute ethanol and dried, and acid-precipitable radioactivity was measured in a scintillation counter, using Cerenkov radiation, at 38% efficiency. Authentic HSV-1 DNA polymerase was obtained from extracts of Vero cells 16 h following high-multiplicity infection with HSV-1 strain 17 (22). Enzyme activity was measured as picomoles of acid-precipitable [35S]MP in 37°C, and 1 unit is defined as 1 pmol/h under these conditions. Concentrations of acyclovir triphosphate (the gift of Brian Terry) were determined by high-pressure liquid chromatography analysis and spectrophotometry.

In vitro transcription and translation. Plasmids derived from pGEM1 or pGEM2 were digested with specified restriction enzymes, and the linearized DNA was purified by phenol extraction and ethanol precipitation from 300 mM sodium acetate. In vitro transcription was carried out with T7 or SP6 RNA polymerase in the presence of ribonucleoside triphosphates, and the RNA was purified by phenol extraction and ethanol precipitation and then sized and quantitated on 1% agarose-formaldehyde gels (16, 21, 28, 35, 41). The RNA was translated in rabbit reticulocyte lysate (Promega BioTech) with or without 20 μCi of [35S]methionine (1,200 Ci/mmol) in a 30-μl volume containing up to 4 μg of RNA at 30°C for 60 to 90 min according to the instructions of the supplier. Aliquots were then taken for assay of polymerase activity or analysis of translation products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) fluorography, using prestained molecular weight markers (Sigma Chemical Co.) (4, 29).

Protein expression in E. coli, purification, and analysis. For expression of the T7-7.1 pol fusion protein in E. coli, plasmid pT7-7.1, carrying an ampicillin resistance marker, was transformed into E. coli HMS174 containing pGP1-2 which carries a kanamycin resistance marker and expresses the T7 RNA polymerase from a lambda phage pl promoter controlled by the heat-sensitive lambda repressor cI587. Transformed cells were selected on LB plates containing both ampicillin and kanamycin at 30°C and then grown, temperature-induced, treated with rifampin (200 μg/ml), and pulse-labeled with [35S]methionine exactly as described by Tabor and Richardson (47). For bulk induction of the fusion protein, cultures of E. coli HMS174 (pGP1-2/pT7-7.1) were grown overnight at 30°C in LB containing ampicillin and kanamycin (both at 50 μg/ml); they were then shaken at 42°C for 20 min and at 37°C for 2 h. Aliquots of pulse-labeled or unlabeled cells were lysed in 0.1 volume of Laemmli sample buffer and analyzed by SDS-PAGE fluorography or SDS-PAGE with Coomassie staining, respectively, on 7.5% Laemmli gels, using prestained molecular weight markers.

Inclusion bodies containing the insoluble T7-7.1 fusion protein were prepared from overnight cultures of E. coli HMS174 (pGP1-2/pT7-7.1) prepared as described for bulk induction, pelleted by centrifugation at 3,000 × g for 10 min, lysed in 20 mM Tris hydrochloride (pH 8.0)–1 mM EDTA containing lysozyme (5 mg/ml) at 0°C for 30 min, and then sonicated for three 20-s cycles at full power with a Branson probe-type sonicator (33, 34). The lysate was then spun at 3,000 × g for 5 min, and the pellet was used as the source of inclusion bodies for analysis.

Production of antiserum. Antiserum to the pT7-7.1-specified pol fusion protein was prepared by immunizing a rabbit with 10 μg of the insoluble material shown in Fig. 6, lane m, in complete Freund adjuvant and boosting three times at 2-week intervals. Antiserum prepared against purified HSV-1 DNA polymerase was provided by Mary Haffey.

Metabolic labeling and immunoprecipitation. Vero cells were mock infected or infected with HSV-1 strain KOS at a multiplicity of infection of 20. At 6 h following infection, cells were pulse-labeled for 45 min in Hanks medium containing 200 μCi of [35S]methionine per ml. Cells were then washed in phosphate-buffered saline and lysed at 4°C in RIPA buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS). Lysates were cleared by Eppendorf centrifugation for 10 min and then incubated with antiserum at a 1:100 dilution for 1 h on ice and then with staphylococcal protein A-agarose (Genzyme; 1:4 of a 50% slurry in water) for another hour on ice. The beads were then washed three times with 5 volumes of RIPA...
buffer and once with 5 volumes of water then incubated with 3 volumes of Laemmli sample buffer at 90°C for 3 min, followed by SDS-PAGE.

RESULTS

Construction of HSV pol templates and in vitro translation. Functional expression of a cloned pol gene in COS-1 cells has been demonstrated through complementation of the tsC7 defect in COS-1 cells transfected with the pol expression vector pD7, also called pD700 (12). The entire pol coding region in pD700 was mobilized on a HindIII-SspI restriction fragment and inserted into pGEM2 between HindIII and HincII such that pol was downstream from the T7 promoter (Fig. 1). Also, two deletion mutants of pol, called 701 (an in-frame deletion of amino acids 881 to 958) and 702 (a deletion of all but 79 base pairs upstream from the pol initiator codon), described further below, were inserted into pGEM2 at the same positions. The pol gene was also inserted into the T7 expression vector, T7-7, as an in-frame fusion at the pol-SnaBI site (deleting the amino-terminal 67 amino acids) downstream from the phage T7 gene 10 initiator, fused at the fourth codon, and called T7-7.1. Similarly, T7-7.2 contains a fusion of the pol gene and T7 gene 10, only at the most proximal pol NaeI site and at the ninth gene 10 codon, resulting in the deletion of the amino-terminal 27 pol amino acid residues.

In vitro transcription of linearized pGEM2 and pT7-7 pol plasmids with T7 RNA polymerase yielded full-length uncapped pol runoff transcripts which could be sized and quantitated on agarose-formaldehyde gels. The in vitro RNA was translated in rabbit reticulocyte lysates in the presence of [35S]methionine for analysis by SDS-PAGE fluorography or in the presence of nonradioactive amino acids for enzyme assays. The major translation product of the full-length pol gene migrates as a 140-kDa polypeptide (Fig. 3A, lane 2). This size corresponds to that of the predicted product of the pol open reading frame (15, 43). The products of deletions 701 (lane 3), T7-7.1 (lane 5), and T7-7.2 (lane 6) could also be distinguished from the full-length pol gene product on a 7.5% Laemmli gel. By linearizing the pol gene template at unique restriction sites within the gene, a nested set of carboxy-terminal deletions was generated (Fig. 2 and 3). The truncated polypeptides migrated accordingly to their predicted sizes; thus, the polypeptide specified by the in vitro translation of pol RNA was colinear with the pol gene itself.

To determine whether the HSV DNA polymerase translated in vitro possesses enzymatic activity, T7 RNA polymerase transcripts of pGEM2-702 were used to program rabbit reticulocyte lysates in the absence of radioactive label and the translation mixture was assayed directly for the presence of HSV DNA polymerase activity. The polymerase activity in the pol RNA programmed reticulocyte lysate was detectable above the background activity found in the reticulocyte lysate (Table 1). The endogenous activity found in the reticulocyte lysate was not significantly altered by varying the salt conditions, but that activity was near the filter background signal detected in the assay. Under similar conditions, E. coli polymerase II Klenow enzyme was inhibited by approximately 50%. No incorporation of acid-precipitable radioactivity above background was detected when pancreatic DNase was added to the enzyme assay.

The incorporation of acid-precipitable radioactivity by the pol-programmed reticulocyte lysate was linear with time up to 20 min of the assay at 37°C (Fig. 4). A reproducible characteristic of the assay was that, at zero time, the reticulocyte lysate programmed with brome mosaic virus RNA or no RNA showed incorporation above background which declined over the time course of the assay. In vitro translated HSV polymerase activity was inhibited by acyclovir triphosphate in a dose-dependent manner (Table 2). Inhibition of the in vitro translated polymerase activity by acyclovir triphosphate was similar to the inhibition of authentic HSV DNA polymerase extracted from HSV-infected Vero cells and added to control rabbit reticulocyte lysate. The in vitro translated activity was inhibited by 50% at 5.5 μM acyclovir triphosphate, the enzyme extracted from HSV-infected Vero cells was inhibited by 50% at 1.2 μM acyclovir triphosphate, and the endogenous DNA polymerase activity in reticulocyte lysate was not inhibited at all at 5 and 10 μM acyclovir triphosphate (Table 2).

Effect of gene deletions on polymerase function. The demonstration of DNA polymerase enzymatic activity in programmed reticulocyte lysates presented an opportunity to study the effects of carboxy-terminal, amino-terminal, and internal deletions of the pol gene. Carboxy-terminal deleted polymerase polypeptides specified by the constructions used as templates for the polypeptides shown in Fig. 3 were assayed and found to lack enzymatic activity (Table 1). The BamHI-pol polypeptide, lacking 164 amino acid residues, is the most distally terminated of the polypeptides examined for polymerase activity. Two amino-terminal deletions, T7-7.1 and T7-7.2, lack 67 and 27 amino-terminal residues, respectively. In vitro transcripts of these constructs specified enzymatically active polymerase (Table 1). The activity specified by T7-7.2 was significantly greater than even the complete pol gene construction pGEM2-702, partially owing to enhanced translational efficiency of pT7-7.2 RNA. The deleted polypeptide specified by pGEM2-701, lacking amino acid residues 881 to 958, possessed no DNA polymerase activity. Construction pGEM2-702 differed from pGEM2-700 only in that it lacked 79 base pairs, including the short upstream open reading frame; however, both specified an enzymatically active polypeptide.

Biological activity of deleted polymerase genes. COS-1 cells transfected with pD700 are able to partially complement the growth defect of an HSV-1 temperature-sensitive DNA polymerase mutant, tsC7 (12). The upstream deletion, pD702, and in-frame deletion, pD701, were examined for biological activity in the complementation assay. COS-1 cells transfected with vectors pD700, pD701, and pD702 expressed approximately equal amounts of pol-specific mRNA detected by Northern (RNA) analysis (data not shown) performed as in a previous study (12). Plasmids pD700 and pD702 were capable of partially complementing the tsC7 growth defect in COS-1 cells at the nonpermissive temperature with nearly equal efficiency (Table 3), while the in-frame deletion, pD701, failed to complement the tsC7 defect. Thus, deletion of the upstream open reading frame region had no detectable effect on expression of a functional pol gene product, while the deleted pol gene product lacking residues 881 to 958 was biologically inactive.

Effects of 5'-proximal deletion on in vitro translation. Short upstream open reading frames are known to regulate expression of several genes at the translational level (21, 24, 31, 36). The construct pD702 lacks the pol short upstream open reading frame, while pD700 has the wild-type sequence. Comparison of the relative in vitro translational efficiencies of these RNAs was made to determine whether the sequence 5' to the pol open reading frame could affect translational efficiency. Uncapped transcripts from pGEM1-702 and
FIG. 1. Construction of plasmids expressing HSV-1 DNA polymerase. Schematic explanation of procedures used to construct expression plasmids by standard techniques. See Materials and Methods and Results.

pGEM1-700 prepared with SP6 polymerase were quantitated on agarose formaldehyde gels and assayed for translational efficiency. The transcripts of pGEM1-702 were more efficiently translated in rabbit reticulocyte lysates than those of pGEM1-700 (Fig. 5). A mixture of equal amounts of RNAs from pGEM1-700 and pGEM1-701 specified equal amounts of both polypeptides, indicating that RNA from pGEM1-700 was not inhibitory for translation of pGEM1-701 RNA (Fig. 5, lane h).

Expression of the pT7-7.1 pol fusion protein in E. coli. Since plasmid pT7-7.1 contains the polymerase gene fused to phage T7 gene 10, the expression system developed by Tabor and Richardson could be used to express the fusion protein in E. coli (47). pT7-7.1 was transformed into E. coli
containing pGP1-2, expressing the phage T7 RNA polymerase from a lambda phage pL promoter controlled by the heat-sensitive lambda phage repressor cI857. Cultures of E. coli containing both pT7-7.1, carrying an ampicillin resistance marker, and pGP1-2, carrying kanamycin resistance, were induced at 42°C with and without the addition of rifampin, and bacterial proteins were pulse-labeled with $[^{35}S]$methionine, displayed by SDS-PAGE, and autoradiographed. pT7-7.1 specified a 135-kDa protein which was expressed in the presence of rifampin (Fig. 6, lane a), indicating that its mRNA was transcribed by T7 RNA polymerase, which is rifampin resistant. The 135-kDa protein was absent from extracts of cells lacking any T7 vector or having pT7-7 with no insert. High-level expression of the 135-kDa pol fusion protein was obtained when stationary cultures of E. coli containing pGP1-2 and pT7-7.1 were induced at 42°C for 20 min and then shifted to 37°C for 1 h (cf. Fig. 6, lanes k and l, for induction of a 135-kDa Coomassie-stained band indicated by an arrow). Although large quantities of the pol fusion protein specified by pT7-7.1 could be produced in E. coli, the protein was soluble only in 8 M urea and ionic detergents. Preparation of inclusion bodies from induced cells yielded a fraction substantially enriched in the insoluble fusion protein (Fig. 6, lane m). Attempts to renature the insoluble pol fusion protein have been unsuccessful.

To confirm the identity of the 135-kDa pol fusion product, the insoluble material displayed in Fig. 6, lane m, was used to prepare rabbit antiserum which precipitates radiolabeled polymerase from extracts of infected cells. The antiserum prepared with the fusion protein precipitated a 140-kDa polypeptide from infected cell extracts and not from uninfected cell extracts (Fig. 7). Furthermore, this 140-kDa polypeptide was identical in mobility to the polypeptide precipitated by antiserum prepared against authentic polymerase purified from HSV-1-infected cells. The antiserum prepared against purified HSV-1 DNA polymerase also precipitated a 65-kDa polypeptide, which probably represents another HSV-1-specified protein known to copurify with the polymerase (42).

### DISCUSSION

In this study, the gene specifying the HSV DNA polymerase was inserted into plasmids, allowing study of the expression of this gene in COS-1 cells and bacteria and by in vitro translation. The polypeptide resulting from in vitro translation of pol RNA possessed DNA polymerase enzymatic activity, and the effects of several gene deletions were examined.

**Deletion of the short upstream open reading frame region.** Approximately 5% of eucaryotic genes studied have short upstream open reading frames, but their role in translational regulation of gene expression has not yet been clearly defined. In some cases, an upstream open reading frame may decrease the translational efficiency of an mRNA by affecting the efficiency of reinitiation at a downstream AUG

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**FIG. 2.** Deletions of the HSV-1 DNA polymerase gene. Schematic drawing showing the HSV-1 DNA polymerase gene, a map of the restriction sites used in the constructions depicted in Fig. 1, and the map locations of the polymerase gene deletions produced in the constructions. Also shown are the predicted molecular weights of carboxy-terminally deleted polypeptides specified by in vitro transcription-translation of pGEM2-702 RNA deleted at the indicated restriction sites. kd, Kilodaltons.

**FIG. 3.** Expression and deletion mapping of the HSV-1 DNA polymerase by in vitro transcription-translation. The indicated templates were linearized at an NdeI or XbaI site distal to the coding region (A) or at restriction sites within the coding regions (B). RNA was obtained by in vitro transcription of the templates, using T7 RNA polymerase (Promega BioTech or New England BioLabs), by previously described methods. The pol RNA was translated in rabbit reticulocyte lysates containing $[^{35}S]$methionine, and the translation products were displayed by SDS-PAGE (7.5%) and fluorography. (A) Lanes: 1, reticulocyte lysate with no RNA; 2, pGEM2-700; 3, pGEM2-701; 4, pGEM2-702; 5, pT7-7.1; 6, pT7-7.2. (B) All from pGEM2-702 linearized at: 1, XbaI (distal to coding region); 2 BamHI; 3, PstI; 4, NotI; 5, AsuII; 6, MstII; 7, SaeI; 8, EcoRI.
TABLE 1. Enzymatic activity of in vitro translated HSV-1 DNA polymerasea

<table>
<thead>
<tr>
<th>Source of:</th>
<th>Salt [100 mM (NH4)2SO4]</th>
<th>Polymerase activity (pmol of [32P]dCTP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA (template)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>0.42</td>
</tr>
<tr>
<td>pGEM-2700</td>
<td>+</td>
<td>2.94</td>
</tr>
<tr>
<td>pGEM-2701</td>
<td>+</td>
<td>0.59</td>
</tr>
<tr>
<td>pGEM-2702</td>
<td>+</td>
<td>5.38</td>
</tr>
<tr>
<td>pT7-7.1</td>
<td>+</td>
<td>5.40</td>
</tr>
<tr>
<td>pT7-7.2</td>
<td>+</td>
<td>9.67</td>
</tr>
<tr>
<td>BamHI</td>
<td>+</td>
<td>0.41</td>
</tr>
<tr>
<td>AscI</td>
<td>+</td>
<td>0.48</td>
</tr>
<tr>
<td>NruI</td>
<td>+</td>
<td>0.41</td>
</tr>
<tr>
<td>PstI</td>
<td>+</td>
<td>0.55</td>
</tr>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reticulocyte lysate</td>
<td></td>
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</tr>
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<td>Reticulocyte lysate</td>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td>pGEM-2702</td>
<td>−</td>
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</tr>
<tr>
<td>pGEM-2702</td>
<td>+</td>
<td>5.35</td>
</tr>
<tr>
<td>HSV/Vero</td>
<td>−</td>
<td>16.40</td>
</tr>
<tr>
<td>HSV/Vero</td>
<td>+</td>
<td>23.55</td>
</tr>
<tr>
<td>Polymerase I Klono</td>
<td></td>
<td>53.98</td>
</tr>
<tr>
<td>Polymerase I Klono + DNaSe I</td>
<td></td>
<td>26.67</td>
</tr>
<tr>
<td>pGEM2702 + DNaSe I</td>
<td>+</td>
<td>0.48</td>
</tr>
</tbody>
</table>

a RNA was obtained by in vitro transcription of linearized templates, purified, and used to program rabbit reticulocyte lysates, using 4 µg of RNA per 30 µl of translation mixture, and nonradioactive amino acids. After incubation at 30°C for 90 min, the entire 30-µl translation mixtures were assayed for DNA polymerase activity by measurement of acid-precipitable radioactivity incorporated by incubation at 37°C for 20 min, using α-[32P]dCTP. For testing sensitivity to salt and DNaSe I, the same assay conditions were used.

codon (21, 24, 31, 36, 39, 40). In the case of the pol gene, the upstream AUG is not placed in a context which would favor its utilization as a translational initiation site, according to the consensus initiation context rules suggested by Kozak (25).

FIG. 4. Time course of DNA synthesis by in vitro translated HSV-1 DNA polymerase. RNA was obtained by in vitro transcription of XbaI-linearized pGEM2-702, using T7 RNA polymerase, and purified by phenol extraction and ethanol precipitation. A 20-µg portion of pol RNA was used to program 150 µl of rabbit reticulocyte lysate translation mixture for 90 min at 30°C, using nonradioactive amino acids. A duplicate sample of rabbit reticulocyte lysate was incubated with no added RNA. The translation mixtures were then assayed for DNA polymerase activity. Aliquots were withdrawn at the indicated times and assayed for acid-precipitable radioactivity as described in Materials and Methods. Symbols: O, pol RNA; □, no added RNA.

TABLE 2. Inhibition of in vitro translated HSV-1 DNA polymerase by acyclovir triphosphate

<table>
<thead>
<tr>
<th>Prepn</th>
<th>% Inhibition by given acyclovir triphosphate conc (µM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>HSV-infected Vero cell extract added to reticulocyte lysate</td>
<td>28</td>
</tr>
<tr>
<td>pGEM-702-programmed reticulocyte lysate</td>
<td>13</td>
</tr>
<tr>
<td>Endogenous activity reticulocyte lysate</td>
<td>0</td>
</tr>
</tbody>
</table>

a The standard reticulocyte lysate translation mixture described in Materials and Methods and Table 1, footnote a, was used. HSV-infected Vero cell extract prepared as described previously (24) was added to reticulocyte lysate and incubated as if for in vitro translation at 1 U of HSV-1 DNA polymerase activity per 30 µl of translation mixture. DNA polymerase inhibition assays were carried out under conditions described in Materials and Methods at the concentrations of acyclovir triphosphate shown.

It would be useful to determine whether the pol upstream open reading frame has an obligatory role in pol gene expression or whether its translation product has any obligatory role in polymerase activity. It is not yet known whether the product of the upstream open reading frame, a decapeptide with the sequence Met-Pro-Ala-Ala-Ser-Leu-Gly-Val-Arg, is actually synthesized by ribosomes in vivo or in vitro. The eucaryotic expression plasmid pD702, containing the BAL31 deletion of the pol upstream open reading frame, was capable of complementing the tsC7 defect to the same extent as the wild-type plasmid, suggesting that there is no obligatory role for the upstream open reading frame region for in vivo pol gene function. Exami-

TABLE 3. Complementation of DNA polymerase mutant tsC7 by transfected plasmids in COS-1 cellsa

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Virus yield (PFU/ml) at:</th>
<th>Complementation index (avg yield, 39°C)b</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>33°C</td>
<td>39°C</td>
</tr>
<tr>
<td>None</td>
<td>1.8 x 10^6</td>
<td>1.2 x 10^3</td>
</tr>
<tr>
<td>pD700</td>
<td>1.3 x 10^6</td>
<td>1.1 x 10^4</td>
</tr>
<tr>
<td>pD701</td>
<td>3.0 x 10^6</td>
<td>0.5 x 10^3</td>
</tr>
<tr>
<td>pD702</td>
<td>3.1 x 10^6</td>
<td>1.1 x 10^4</td>
</tr>
</tbody>
</table>

a COS-1 cells (5 x 10^6) were transfected with plasmid and carrier DNA by the DEAE-dextran technique. At 48 h following transfection, the cells were infected with tsC7 at 37°C for 1 h at a multiplicity of 5. Following adsorption, the cell monolayers were washed five times with serum-free medium and refed, and virus was harvested at 18 h postinfection. Virus yields were determined by titration on Vero cells at 33°C.

b The complementation index was calculated by dividing the yield of the mixed infection at the permissive temperature assayed at the permissive temperature by the same yield from cells transfected with no plasmid.
nation of in vitro translation of pol RNA revealed that wild-type RNA is less efficiently translated than RNA with the 702 deletion in reticulocyte lysates, which are capable of downstream initiation (27, 38). It appears, therefore, that pol RNA lacking the upstream open reading frame region has enhanced translational capabilities, at least in vitro. Experiments with in vitro capped pol RNA have shown similar results (data not shown), but unlike the situation for some other messages, capping does not appear to significantly alter in vitro translation efficiencies of pol RNA (41). The mechanism for the apparent enhancement of translation of pol RNA lacking the upstream region is not evident. It is possible that the secondary structure of the region, and not the open reading frame itself, may prove to be critical, since the 5'-proximal portion of the pol mRNA is rich in potential stable secondary structures which may be capable of attenuating translation (26, 41). The initiator codon of the large pol open reading frame is in the stem of a hairpin with a predicted free energy of −71 kcal (ca. −297 KJ), and the upstream open reading frame is within this potential structure (19). The BAL31 deletion in pD702 effectively breaks up this secondary structure, and that may account for the enhancement of translation in vitro. Therefore, further work must be done to distinguish between these possible explanations for how this deletion affects translation. Although no diminished complementation was observed for the pD702 construct in vivo, translational regulation may limit the amount of the pol gene product relative to other early viral gene products.

Enzymatic activity of the in vitro translated product. HSV-1 DNA polymerase activity copurifies with the 140-kDa polypeptide purified to homogeneity and demonstrated to be capable of processive replication of single-stranded DNA templates (37). The primary translation product of the RNA specified by the cloned pol gene has been shown in this work to be a polypeptide of 140 kDa and colinear with the restriction map of the pol gene (Fig. 2 and 3). The 140-kDa polypeptide specified by the large pol open reading frame possessed DNA polymerase enzymatic activity when assayed by standard methods on an activated calf thymus DNA template. The in vitro translated pol enzymatic activity was salt resistant, much like the authentic virus-specified enzyme in crude extracts, and incorporation of acid-precipitable radioactivity was removed by the action of DNase I (Table 1). The in vitro synthesized polymerase was also sensitive to inhibition by acyclovir triphosphate, and comparable inhibition of HSV polymerase extracted from HSV-infected Vero cells was observed, consistent with earlier observations on the sensitivity of HSV-1 DNA polymerase to acyclovir triphosphate in crude extracts (Table 2) (14). While the sensitivity profile of the in vitro translated poly-
merase was not identical to that of the infected cell extract activity, the in vitro translated product was considerably more sensitive to the inhibitor than was the endogenous reticulocyte activity. That the in vitro translated pol gene product possessed enzymatic activity confirms the previous studies establishing that pol enzymatic activity resides in the 140-kDa polypeptide (37, 42) but also shows that no additional viral gene products are necessary to obtain core polymerase activity (nucleotide incorporation into an activated calf thymus DNA template) through either direct participation or any obligatory modification of the 140 kDa polypeptide. Expression of the pol open reading frame as a fusion protein in E. coli resulted in an insoluble 135 kDa polypeptide. Antiserum prepared against this 135-kDa pol fusion polypeptide precipitated a 140-kDa polypeptide only from HSV-1-infected cells which comigrated with the polypeptide precipitated by anti-serum prepared against HSV-1 DNA polymerase isolated from infected cells, confirming that the 135-kDa pol fusion protein contains HSV-1 DNA polymerase antigenic determinants. Thus, the open reading frame expressed in pD702, pGEM2-702, and their relatives was that of the HSV-1 DNA polymerase.

Effect of gene deletions on polymerase activity in vivo and in vitro. The detection of DNA polymerase enzymatic activity by in vitro transcription-translation of cloned HSV DNA pol genes allows deletion mapping of the activity within the gene. This study established that enzyme activity is not detectable in a polypeptide lacking the carboxy-terminal 164 residues, corresponding to the proximal BamHI site. None of the other carboxy terminally truncated polypeptides possess any detectable enzymatic activity; therefore, the carboxy terminus of the polypeptide, within the last 164 residues, is indispensable for enzyme activity. The in-frame deletion mutant 701 also lacks enzymatic activity, and this correlated with its lack of ability to complement the tsC7 growth defect, indicating its lack of biological activity. The deletion 701 spans the region 881 to 895, which is highly conserved among the homologous polymerases of Epstein-Barr virus, human cytomegalovirus, varicella-zoster virus, vaccinia virus, adenovirus type 2, PRD1, T4, and the human alpha DNA polymerase (1, 15, 20, 30, 43, 48). The lack of activity of mutant 701 supports the hypothesis that the highly conserved region of the polymerase polypeptide plays an important role in catalytic function. The role of this region in polymerase function is at this time unknown. Secondary-structure modeling of this region of the polypeptide by computer application of the Chou-Fasman algorithm predicts a beta sheet-turn-beta sheet structure flanked by two long alpha-helical regions; this structure does not resemble any known nucleotide- or DNA-binding protein motif (7).

Two amino-terminal deletions of pol (27 and 67 residues), expressed as fusions with several amino-terminal residues of phase T7 gene 10, specified polypeptides by in vitro translation which possesses greater enzymatic activity than the product of the full-length pol gene. One factor which can account for only a small part of this difference is that the RNAs specified by pT7-7.1 and pT7-7.2 are more efficiently translated in vitro than the pGEM2-702 RNA, but by less than a factor of 2 (data not shown). The severalfold enhancement observed may be due to the removal of a portion of the polypeptide which requires interaction with other viral or host cell polypeptides in order to sustain enzymatic activity. Alternatively, removal of the amino terminus of pol may diminish the capacity for processive activity when containing nonprocessive, or distributive, activity of the polypeptide.

By further examining the in vitro enzymatic activities of polypeptides generated by site-directed in vitro mutagenesis of the pol gene, a detailed functional domain map of this enzyme can now be obtained.

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LITERATURE CITED


