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# Translational Regulation of Herpes Simplex Virus DNA Polymerase

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**Using as antigens fusion proteins expressed in bacteria, we have generated polyclonal antisera specific for the herpes simplex virus (HSV) type 1 DNA polymerase. A variety of immunologic, genetic, and biochemical assays were used to characterize these antisera and demonstrate their specificity for the HSV DNA polymerase. Using these antisera, measurements of the synthesis and accumulation of HSV DNA polymerase in infected Vero cells were made. Peak rates of polymerase synthesis were observed at 4 h postinfection, as much as 2 h before peak levels of polymerase mRNA accumulation. At all times examined, the HSV DNA polymerase polypeptide was found to be synthesized at a lower rate per mRNA than the viral thymidine kinase, with this difference being especially dramatic at later times. Infected-cell RNA isolated at 2 and 6 h postinfection directed the synthesis of similar amounts of polymerase polypeptide per polymerase transcript in rabbit reticulocyte lysates, indicating that polymerase transcripts are inherently as translatable at both times. An HSV mutant in which sequences including a short upstream open reading frame in the HSV DNA polymerase transcript were deleted specified polymerase mRNA whose translational efficiency was no more than marginally greater than that of the wild-type virus. These results demonstrate that polymerase expression is regulated by inefficient translation mediated by sequences other than the short upstream open reading frame and that this leads to an early shutoff of polymerase synthesis during HSV infection.**

Herpes simplex virus (HSV) encodes a DNA polymerase (Pol) which is required for viral DNA replication (49). The *pol* locus was initially defined by studies using temperature-sensitive *pol* mutants (3, 4). Subsequent DNA sequencing and RNA mapping studies established that a 4.2-kilobase polyadenylated mRNA containing an open reading frame (ORF) coding for a 137-kilodalton polypeptide is transcribed from this region (11, 16, 39, 40, 50). As might be expected for a transcript coding for a protein involved in viral DNA replication, transcription and accumulation of *pol* transcripts have been shown to possess delayed-early kinetics (16, 46, 50, 51).

Measurement of transcription rates by pulse-labeling intact cells and with nuclear run-on reactions has established that *pol* mRNA is synthesized at a rate comparable to that of several delayed-early viral genes, including those coding for thymidine kinase (*tk*) and the major DNA-binding protein (*dbp* or ICP8) (46, 51). However, Pol is not as abundant as TK or ICP8 in infected cells, suggesting that *pol* expression is subject to posttranscriptional regulation. Transcripts for Pol are relatively abundant in the cytoplasm of infected cells; however, unlike other delayed-early transcripts, only a small fraction are associated with large polysomes at 6 h postinfection (50). This further indicates that *pol* expression is regulated to some extent at the level of translation. We and others have raised the hypothesis that this regulation could be due to the presence of a short ORF upstream of the *pol* major ORF (7, 50).

To examine the regulation of *pol* expression, Pol-specific antisera were generated by using β-galactosidase-Pol fusion proteins. Immunological, biochemical, and genetic methods were used to demonstrate that the sera generated against the fusion proteins are specific for Pol. Using these antisera, we show that regulation of Pol synthesis differs from that of another HSV delayed-early polypeptide, leading to early shutoff of Pol translation, and have examined the role of the upstream ORF in translational efficiency.

## MATERIALS AND METHODS

**Cells and viruses.** African green monkey kidney (Vero) cells were grown and maintained as described previously (50). HSV type 1 strain KOS served as the wild-type virus (50). The DP6 cell line was established by stable transformation of Vero cells with sequences containing *pol*. The construction of this cell line and the deletion mutant viruses ΔS1 and ΔB10 are described in the accompanying article (28).

**Plasmid construction.** Plasmids used to generate fusion proteins were maintained in *Escherichia coli* X90 [*ara* Δ(*lac-pro*)*nalA argE* (Am) *thi* Rif$^r$ F′ (*lac*$^+$ *pro*$^+$*lacI*$^{q1}$)] cells (33). A 1.3-kilobase-pair *Sal*I fragment, coordinates 0.432 to 0.445, containing sequences representing 261 amino acids of the carboxyl terminus of the predicted *pol* ORF was isolated from pSG17 (12) and inserted in frame at a unique *Sal*I site near the 3′ end of the *lacZ* gene in pUR290 (42), generating pLacZ-pol4D (see Fig. 1). A second plasmid, pLacZ-polBP, was constructed by excising a 2.3-kilobase-pair *Bgl*II-*Pst*I fragment, coordinates 0.418 to 0.432, containing sequences representing 785 (223 to 1008) amino acids of Pol from the plasmid designated pDP1 (28) and inserting it in frame near the 3′ end of *lacZ* in pUR291 (Fig. 1) (42).

**Preparation of Pol antisera.** X90 cells containing the plasmid constructs were grown in Luria broth (26) to an optical density at 550 nm of 0.25. Fusion protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.5 mM. After 3 h, the cells were harvested by centrifugation, washed with phosphate-buffered saline, and lysed by being boiled in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer. Samples were sonicated, clarified by centrifugation (10,000 × *g* for 10 min), and fractionated on SDS-polyacrylamide gels (22). Proteins were visualized by soaking the gels in 4 M sodium acetate (15), and gel fragments containing the

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fusion proteins were emulsified with an equal volume of Freund complete adjuvant. New Zealand White rabbits were immunized by subcutaneous injections at several sites with a total of 250 μg of protein. Booster injections with 100 μg of protein in Freund incomplete adjuvant were given at monthly intervals. Immunization of rabbits with fusion proteins expressed from pLacZ-pol4D and pLacZ-polBP generated the PP5 and BGG4 sera, respectively.

**Analysis of HSV-infected-cell polypeptides.** For immunoblot analysis, proteins from infected Vero cells were extracted and electrophoresed through SDS-polyacrylamide gels. The proteins were transferred electrophoretically to nitrocellulose filters, as described by Towbin et al. (45). The nitrocellulose filters were blocked overnight in TBST (10 mM Tris chloride [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 1% bovine serum albumin, followed by incubation for 60 min at room temperature with antisera diluted 1:1,000 in TBST. After a washing, bound antibodies were located by incubation with immunoglobulin conjugated with alkaline phosphatase (Promega Biotec) according to the instructions of the manufacturer.

Immunoprecipitation analysis was performed on lysates of $2 \times 10^5$ HSV-infected Vero cells pulse-labeled for 20 min with 200 μCi of [$^{35}$S]methionine per ml (1,072 Ci/mmol; New England Nuclear Corp.) in Earle basal medium minus methionine. Cell monolayers were scraped into cold phosphate-buffered saline and lysed in 100 μl of lysis buffer (10 mM Tris chloride [pH 7.5], 50 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 0.1% sodium azide). The cell lysate was sonicated briefly, made 1% SDS and 150 mM β-mercaptoethanol, incubated for 7 min at 90°C, diluted with 9 volumes of lysis buffer containing 150 mM NaCl, and clarified by spinning at 10,000 × *g* for 30 min. The lysates were preincubated for 30 min at 4°C with normal rabbit serum and 10% protein A–Sepharose CL-4B (Sigma Chemical Co.) and then reacted with 5 μl of Pol antiserum or 1 μl of TK-specific serum (a gift from W. P. Summers) and 100 μl of 10% protein A-Sepharose CL-4BB for 8 h at 4°C. The immunoprecipitates were washed sequentially with RIPA buffer (lysis buffer with 150 mM NaCl and 0.1% SDS), HSB (10 mM Tris [pH 7.5], 2 M NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium azide), RIPA buffer without SDS, and finally with 10 mM Tris chloride (pH 7.5). The precipitates were suspended in SDS-polyacrylamide gel electrophoresis sample buffer and run on SDS-polyacrylamide gels (22). Following electrophoresis, the gels were fixed, processed for fluorography (2), and exposed to preflashed XAR-5 film (Eastman Kodak Co.) (24). Autoradiograms were scanned by laser densitometry (LKB Instruments, Inc.). The levels of protein synthesis were normalized for differences in the predicted methionine content of the two proteins (16 methionines for TK, 22 for Pol).

**DNA polymerase assay.** Crude nuclear extracts were prepared from mock and HSV-infected cells at 16 h after infection as described by Elias et al. (8). To assay DNA polymerase activity, reaction mixtures (50 μl) contained 7.5 μg of activated salmon testes DNA, 63 μM each dATP, dGTP, and TTP, 9 μM [α-$^{32}$P]dCTP (100 Ci/mmol), 20 mM Tris chloride (pH 7.5), 0.1 mM EDTA, 3 mM $MgCl_2$, 5 mM dithiothreitol, 4% glycerol, 40 μg of bovine serum albumin per ml, 150 mM $(NH_4)_2SO_4$, 5 to 10 μl (1.3 mg/ml) of crude extract, and 1.25 mg of dialyzed serum. DNA polymerase activity was determined by measuring trichloroacetic acid-precipitable radiolabel after 30 min at 37°C.

**Isolation and expression of *pol* mRNA.** Cytoplasmic RNA was prepared from Vero cells by lysis with nonionic detergents as previously described (50). The relative levels of *pol* and *tk* transcripts were determined by quantitative nuclease protection assays (50).

Polyadenylated RNA was isolated by chromatography on oligo(dT)-cellulose (Collaborative Research, Inc.) according to the protocol provided by the manufacturer, with the exception that all solutions were buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-NaOH in place of Tris hydrochloride. For Northern (RNA) blot hybridization, polyadenylated RNA was separated on agarose gels containing 0.66 M formaldehyde, 20 mM MOPS (morpholinepropane-sulfonic acid, pH 7.0), 5 mM sodium acetate, and 0.5 mM EDTA and transferred to GeneScreen Plus (Dupont, NEN Research Products). The RNA was immobilized to the filter by cross-linking with UV light. The filter was prehybridized for 2 h at 65°C in 2× SSPE (0.18 M NaCl, 10 mM $NaH_2PO_4$, 1 mM EDTA [pH 7.4])–5× Denhardt solution–1% SDS and then hybridized for 16 h in the same solution containing random-primed labeled KOS *Bam*HI fragment Q (coordinates 0.413 to 0.436). The filter was washed twice for 15 min with 2× SSPE–1% SDS at room temperature and twice for 30 min with 0.2× SSPE–0.1% SDS at 65°C.

Polyadenylated RNAs were translated in a commercial rabbit reticulocyte lysate (Dupont, NEN) according to the protocol of the manufacturer. Reactions were performed with amounts of polyadenylated RNA determined to be nonsaturing. Polyadenylated RNAs containing equivalent amounts of *pol* transcripts were added to lysate containing [$^{35}$S]methionine and incubated for 60 min at 37°C. The reactions were stopped by adding 7 volumes of phosphate-buffered saline and clarified by centrifugation (100,000 × *g* for 30 min). Pol polypeptides were immunoprecipitated and analyzed as described above.

## RESULTS

**Production of antisera.** For the purposes of generating Pol-specific antisera, two gene fusions were prepared and expressed in *E. coli*. Vectors pUR290 and pUR291 are high-copy-number plasmids containing several unique endonuclease restriction sites near the carboxyl terminus of *lacZ* (42). On the basis of the DNA sequence of the predicted HSV strain KOS *pol* ORF (11), fragments were inserted in frame at the carboxyl terminus of the *lacZ* gene. One construct, placZ-pol4D, contains sequences corresponding to the 261 amino acids (974 to 1235) of the carboxyl terminus of Pol (Fig. 1). These sequences lie outside the major regions of DNA polymerase sequence similarity (10, 23, 48). A second plasmid, placZ-polBP, was also constructed by using sequences corresponding to the central 785 amino acids (223 to 1008) of Pol (Fig. 1). Figure 2 shows an example of the abundant levels of IPTG-induced fusion protein synthesis in cells containing pLacZ-Pol4D and pLacZ-PolBP. The heterogeneous migration of the fusion proteins is probably the result of limited proteolysis. Gel-purified proteins were used to immunize rabbits.

**Neutralization of Pol activity in infected-cell nuclear extracts.** The antisera generated against either fusion protein were examined by using an in vitro functional assay (8). Pol activity in partially purified infected-cell extracts was shown to be dependent on the presence of an activated DNA template, to have an enhanced activity in the presence of 150 mM $(NH_4)_2SO_4$, and to be inhibited by phosphonoacetate (results not shown) (27, 38). The effects of PP5 and BGG4

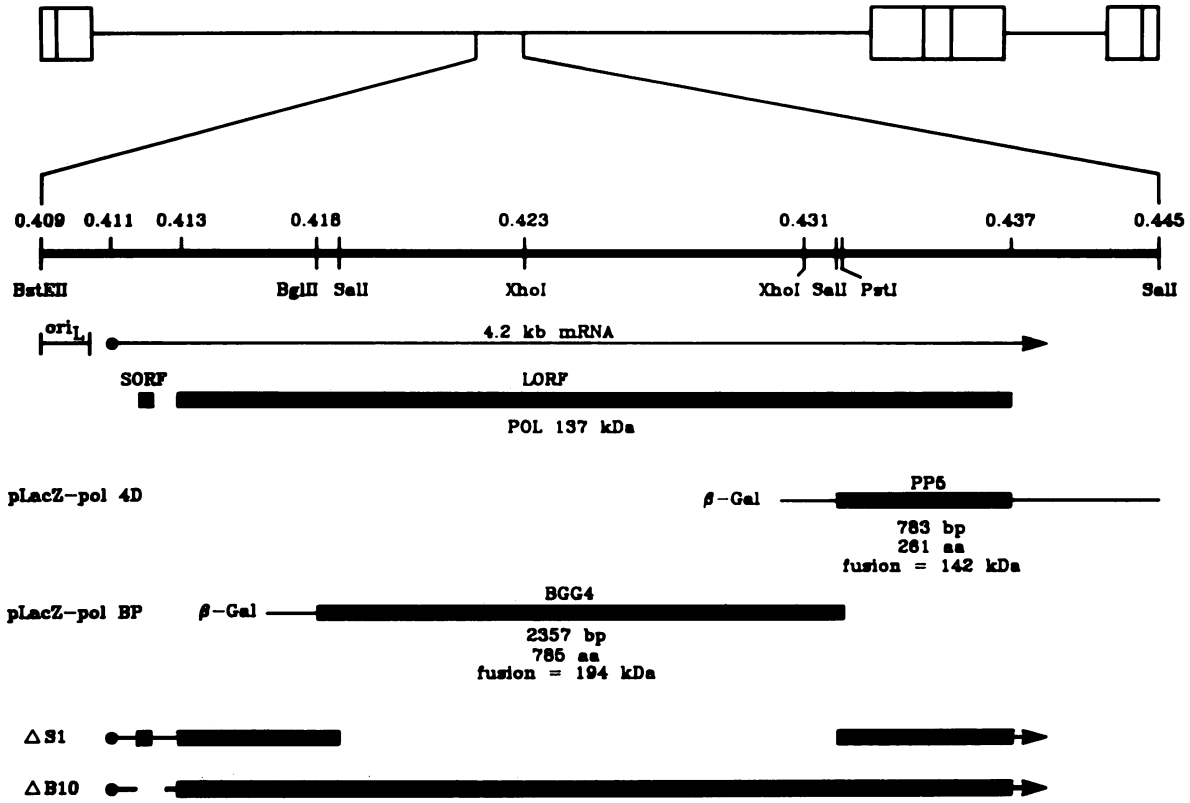


FIG. 1. Construction of *lacZ-pol* plasmids and recombinant viruses. The locations of the *pol* transcript and ORF are presented, along with a few relevant restriction endonuclease sites. Sequences used for the fusion constructs placZ-polBP and placZ-pol4D and the viral mutants ΔS1 and ΔB10 are shown. aa, Amino acids; kDa, kilodaltons; SORF, short ORF; LORF, long ORF; bp, base pairs.

sera on this activity in infected-cell extracts are presented in Fig. 3. The total amount of serum protein was brought to 1.25 mg by the addition of normal rabbit serum where needed. Both PP5 and BGG4 inhibited Pol activity. It is interesting that the PP5 serum which was raised against the 261 amino acids of the carboxyl terminus, which are outside the major regions of DNA polymerase sequence similarity (23), inhibited Pol activity to a similar extent as did BGG4. This suggests that this region may be important for the function of the enzyme.

**Genetic evidence demonstrating the specificity of the Pol antisera.** On the basis of DNA sequencing (11, 39) and molecular mass estimates of partially purified preparations (6, 38), the Pol polypeptide has a predicted molecular mass of approximately 137 kilodaltons. To determine whether PP5 and BGG4 were specific for a protein of this size, Vero cells infected with wild-type virus were incubated with [$^{35}$S]methionine from 2 to 6 h postinfection and lysed, and the radiolabeled proteins were analyzed by immunoprecipitation. A mixture of PP5 and BGG4 immunoprecipitated a single predominant ca. 137-kilodalton polypeptide from lysates of cells infected with wild-type strain KOS (Fig. 4, left, lane KOS). Similar results were obtained with either antiserum singly in immunoprecipitation or Western (immuno-) blot analyses, and neither reacted detectably with proteins from uninfected lysates (results not shown).

The HSV recombinant ΔS1 contains a 3.0-kilobase-pair in-frame deletion in the *pol* gene (Fig. 1) (28). Vero cells infected with this mutant would be expected to express a 57-kilodalton Pol polypeptide. When the mixture of PP5 and BGG4 was used, immunoprecipitation of a 137-kilodalton polypeptide from lysates of cells infected with ΔS1 was not observed. Instead, a 57-kilodalton polypeptide was precipitated (Fig. 4, left, lane ΔS1), in excellent agreement with the size predicted for Pol in cells infected with ΔS1. As a control for infection, a portion of each lysate was precipitated with antiserum specific for the viral TK (Fig. 4, right, lanes ΔS1 and KOS). On the basis of these results, we can conclude that PP5 and BGG4 react specifically with Pol and not with another polypeptide of similar size.

**Expression of the Pol polypeptide.** Having demonstrated their specificity, we began to use these antisera to examine Pol expression. To examine the kinetics of Pol synthesis, immunoprecipitation analysis was performed on lysates from cells pulse-labeled at different times postinfection. The results of this analysis are shown in Fig. 5A. Synthesis of Pol was detectable at 2 h postinfection, peaked at around 4 h, and then declined to quite low levels by 9 h. Immunoblot analysis of lysates at similar times postinfection also showed detectable levels of this polypeptide at 2 h postinfection (Fig. 5B). The accumulation of Pol reached peak levels at 6 h, and this level was maintained at least to 12 h after infection. The persistence of accumulated levels of Pol at late times despite a greatly reduced rate of synthesis indicates that Pol is a stable protein. This finding is consistent with the interpretation of pulse-chase analyses that have been recently reported (43).

**Comparison of the relative rates of Pol and TK synthesis.** We have previously demonstrated that during the peak time of *pol* mRNA accumulation (6 h postinfection), relatively few *pol* transcripts are found associated with large polyribosomes (50). One likely interpretation for this observation is

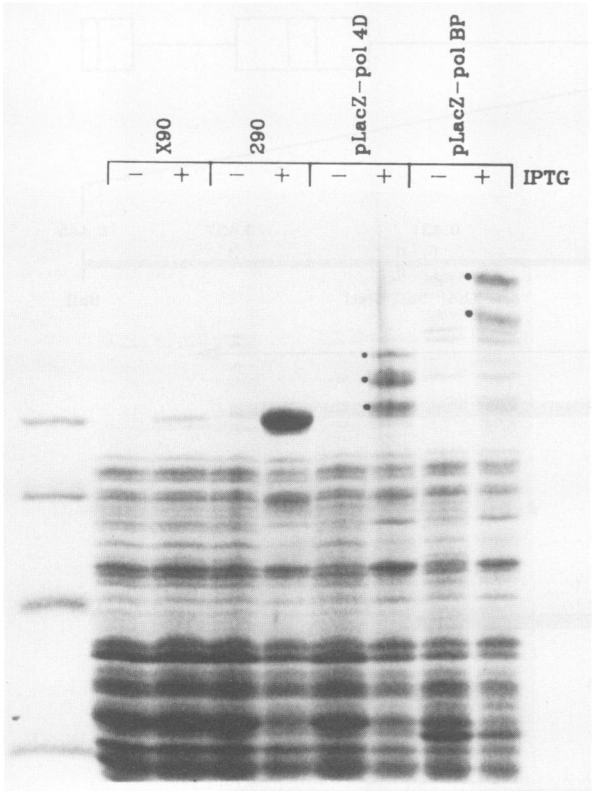


FIG. 2. Fusion protein production. Coomassie blue-stained gel showing inducible expression of fusion constructs in *E. coli*. X90 is the *lacI*$^{q1}$ strain of *E. coli* used as the host for all constructs. 290 is an X90 cell line carrying the parent plasmid pUR290. Dots have been placed alongside the heterogeneously migrating fusion proteins produced in cells carrying placZ-pol4D and placZ-polBP after induction with IPTG. Molecular masses (in kilodaltons) are indicated at the left.

that translation of *pol* transcripts is inefficient. To test this, the anti-Pol sera were used to make a quantitative comparison of Pol synthesis with another delayed-early viral polypeptide, TK. At 6 h following infection, the accumulated levels of *tk* and *pol* transcripts are similar (50). Accordingly, any differences in synthesis of TK and Pol should be a reflection of the relative translational efficiencies of their transcripts. Vero cells infected for 6 h were pulse-labeled for 20 min with [$^{35}$S]methionine, and the level of radiolabel incorporated into Pol and TK was determined by immunoprecipitation. To ensure that this assay was quantitative, lysates were subjected to three rounds of incubation with antisera. No TK or Pol polypeptides were detected in the third precipitation. The immunoprecipitated material was pooled and analyzed by SDS-polyacrylamide gel electrophoresis. TK antiserum precipitated a predominant polypeptide of approximately 43 kilodaltons (Fig. 6, lane 1). PP5 immunoprecipitated the predicted 137-kilodalton Pol (Fig. 6, lane 2). The material immunoprecipitated by a mixture of PP5 and anti-TK (Fig. 6, lane 3) was quantitated by laser densitometry scanning, and the results were normalized for differences in the methionine content, yielding a molar ratio of TK to Pol synthesis of 7:1 (Table 1). When relative mRNA levels are also taken into account, the molar ratio of TK and Pol synthesis per transcript is 6:1 (Table 1). Comparison of Pol and ICP8 synthesis yielded similar results (not shown). These results are consistent with inefficient translation of *pol* transcripts.

**Effect of deleting sequences containing the short ORF on Pol synthesis.** The Pol AUG initiation codon is 209 nucleotides downstream from the 5′ (+1 nucleotide) cap structure (11, 50). Beginning 127 nucleotides upstream from the *pol* initiation codon is a short ORF (nucleotides +82 to +115) that could code for a decapeptide (11, 39). There is a large body of evidence indicating that the presence of short upstream reading frames can influence translation of both cellular and viral mRNAs (9, 17, 18, 20, 29). To determine whether sequences containing this upstream ORF might affect *pol* expression, we analyzed *pol* mRNA and protein synthesis in a virus, ΔB10, lacking nucleotides +57 to +200 (Fig. 1), a deletion which completely removes the upstream ORF from the *pol* transcript.

Relative to *tk* mRNA levels, the levels of *pol* transcripts in cells 6 h postinfection were nearly identical in cells infected with KOS or ΔB10 (Table 1). To measure Pol synthesis, immunoprecipitation analysis was performed on pulse-labeled lysates also at 6 h postinfection (Fig. 6, lanes 4 to 6). In cells infected with ΔB10, the ratio of TK to Pol synthesis per transcript was 4:1, indicating that Pol expression was at most only marginally greater in cells infected with the mutant (Fig. 6, lanes 4 to 6; Table 1).

**Temporal regulation of Pol translation.** The peak rate of Pol synthesis occurred at 4 h postinfection (Fig. 5). This result is in contrast to our previous observation that accumulation of *pol* transcripts did not reach peak levels until as much as 2 h later (50). This suggested that the translation of *pol* transcripts is subject to temporal control. To determine whether this was indeed the case, a comparison was made of *pol* and *tk* transcript levels and their translation products at different times after infection.

At different times after infection, cells were briefly pulse-labeled with [$^{35}$S]methionine; samples of these labeled cells were lysed, and levels of TK and Pol synthesis were determined by immunoprecipitation. The remaining cells were used to quantitate *tk* and *pol* transcript levels via nuclease protection assays. The results obtained from these analyses are presented in Fig. 7. As expected, the peak levels of *pol* transcript accumulation did not correspond to the peak levels of Pol synthesis (Fig. 7, top). In contrast, there was a much closer correspondence of *tk* transcript accumulation and its translation (Fig. 7, bottom), indicating that the observed decline in the efficiency of translation of *pol* transcripts is not a general phenomenon of delayed-early mRNAs. Comparison of the molar ratio of TK to Pol synthesis on a per-transcript basis reveals that in this experiment the relative efficiency of Pol translation changed from approximately 3:1 from 2 to 4 h postinfection to equal to or greater than 7:1 after 4 h postinfection, with a value greater than 18:1 at 6 h postinfection.

This decline does not appear to be the result of changes in the *pol* transcript. On the basis of S1 nuclease analysis, there were no observable differences in the major 5′-end-labeled products protected by *pol* transcripts at early and late times (50). In addition, polyadenylated RNAs containing equivalent amounts of *pol* transcripts (based on Northern blot hybridization) isolated from cells at early and late times during infection (Fig. 8, left) were translated equally well into immunoprecipitable Pol by rabbit reticulocyte lysates (Fig. 8, right).

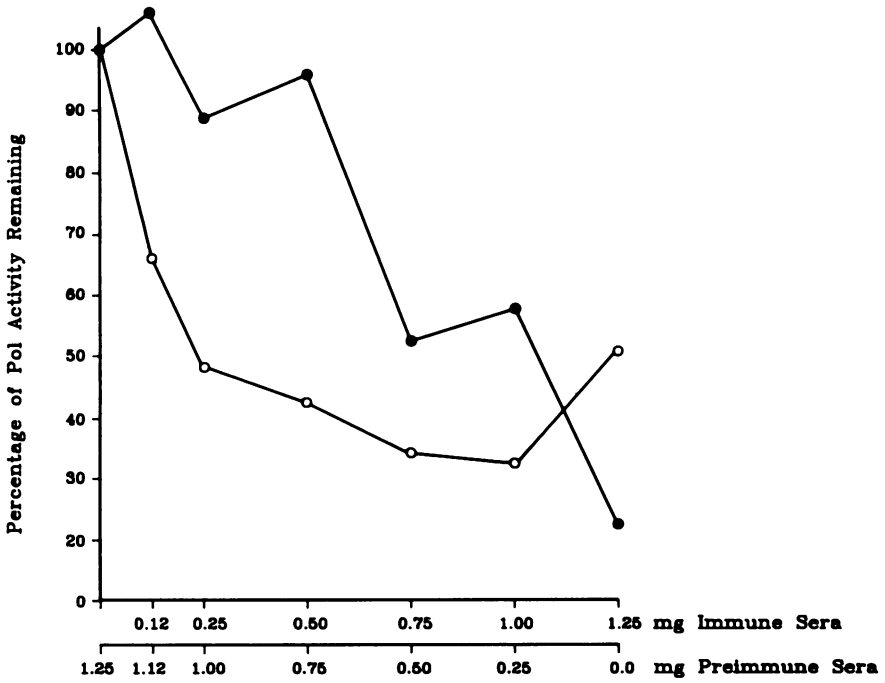


FIG. 3. Neutralization of HSV Pol activity. Partially purified infected-cell nuclear extracts (8) were incubated with activated DNA, deoxynucleoside triphosphates, and increasing amounts of anti-Pol sera. Preimmune serum was added to maintain a constant level of serum protein in each reaction mixture. After 30 min at 37°C, acid-precipitable counts were determined, and the results from a typical experiment performed in duplicate are presented as percentages of DNA polymerase activity remaining, with activity in reaction mixtures containing only preimmune serum being 100%. The percentage activity remaining after incubation with PP5 (●) and after incubation with BGG4 (○) is shown.

## DISCUSSION

We report in this article experiments, using specific antisera to identify the HSV DNA polymerase polypeptide, to determine that Pol expression is inefficient at the level of translation and to characterize Pol expression during HSV infection.

**Specificity of Pol antisera and identification of the Pol polypeptide.** For our studies, we raised antisera against two β-galactosidase-Pol fusion proteins. To ensure specificity, care was taken to exclude substantial amounts of any other HSV ORF from our plasmid constructions and to purify the polypeptides before immunization. The antisera generated recognized only a single predominant species of about 137 kilodaltons in Western blots of infected cells (e.g., Fig. 5B), which was not detected in mock-infected cells. Similarly, under appropriate conditions, the most predominant species by far that was immunoprecipitated by either antiserum was about 137 kilodaltons (e.g., Fig. 4, 5A, 6, and 8).

During the preparation of this manuscript, several laboratories reported the generation of antisera designed to react with Pol (7, 13, 31, 43, 47). These antisera were generated by immunization with Pol partially purified from HSV-infected cells (13, 43), immunization with peptides derived from the predicted sequence of the *pol* gene (43), and immunization with polypeptides expressed in *E. coli* (7, 31, 47). All of these antisera react with a predominant polypeptide of approximately 137 kilodaltons; however, several also react with one or more other prominent polypeptides (7, 43). Pol migrates in a region of SDS-polyacrylamide gels containing several other cellular and viral polypeptides; therefore, we have in addition used a genetic approach to validate the specificity of our antisera. The demonstration that the immunoreactive species expressed by the *pol* deletion mutant ΔS1 exhibited

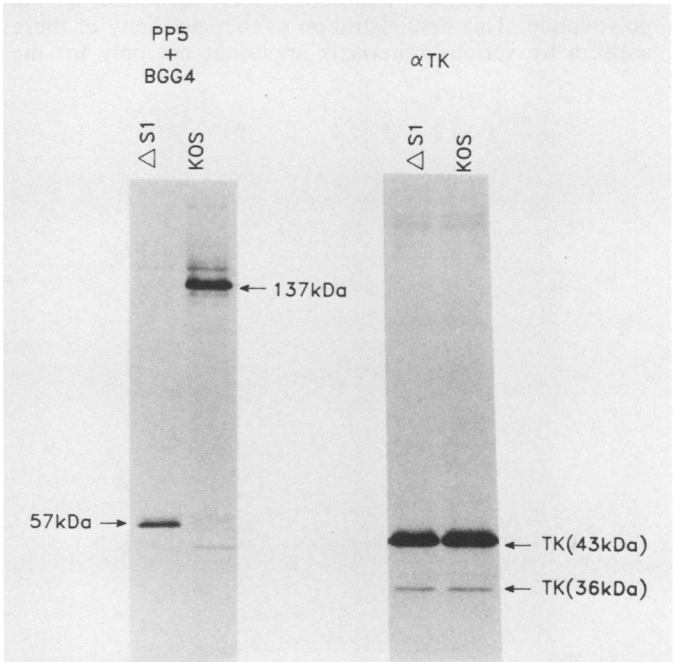


FIG. 4. Immunoprecipitation of a full-length and a truncated Pol polypeptide. (Left) Vero cells infected with the *pol* deletion mutant ΔS1 or wild-type virus (KOS) were pulse-labeled at 2 to 6 h postinfection with [$^{35}$S]methionine. Cells were lysed, precleared with preimmune serum, and then incubated with a mixture of PP5, BGG4, and protein A-Sepharose. Washed immune precipitates were than analyzed by SDS-polyacrylamide gel electrophoresis. (Right) Immunoprecipitation with anti-TK (αTK) serum served as a control for infection with both viruses (ΔS1 and KOS, wild type). In both lysates, anti-TK serum precipitated a major 43-kilodalton (kDa) and a minor 36-kilodalton species.

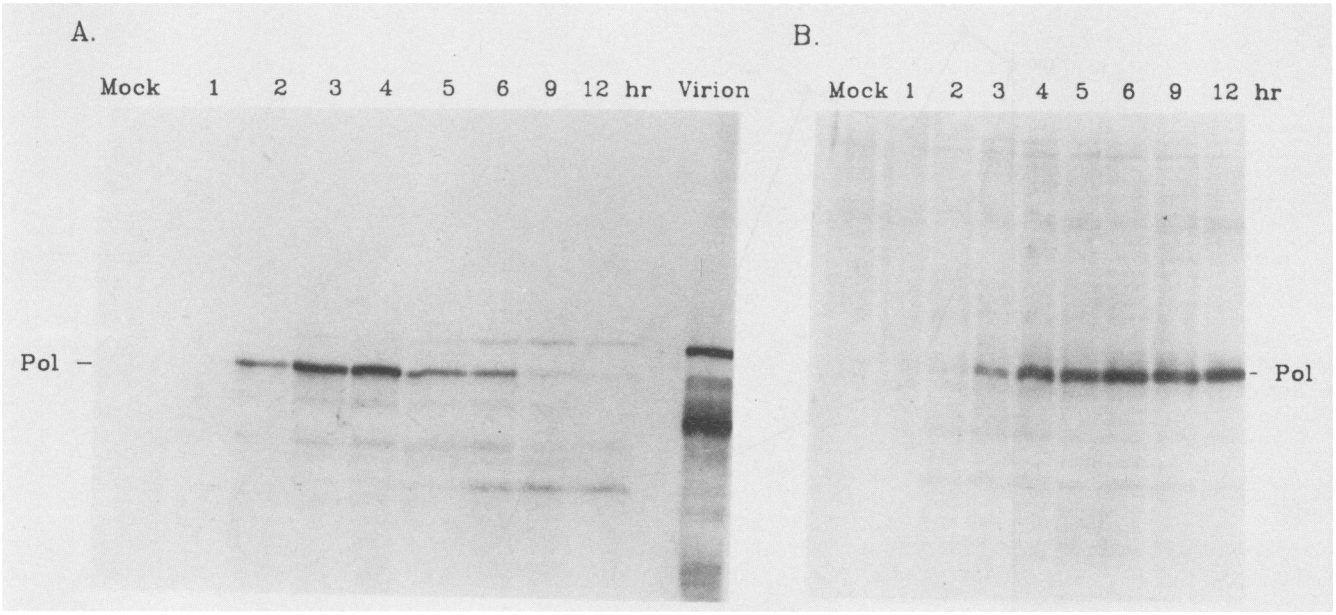


FIG. 5. Time course of HSV Pol synthesis and accumulation. (A) Autoradiograph of immunoprecipitated [$^{35}$S]methionine-labeled protein. Vero cells were labeled for 20 min at different times following HSV infection. Precleared cell lysates were incubated with PP5 and protein A-Sepharose. Immunoprecipitates were then washed and analyzed by SDS-polycarylamide gel electrophoresis. (B) Immunoblot of Pol polypeptide present at various times postinfection. Extracts of cells mock infected or infected with HSV for different times were electrophoresed on SDS–7.5% polyacrylamide gels and electroblotted to nitrocellulose. The blot was probed with PP5 and goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase and developed according to the protocol of the manufacturer.

the expected molecular mass (Fig. 4) established the specificity of these antisera and conclusively identified the Pol polypeptide. This demonstration of the specificity of these antisera by various criteria is important not only for the experiments described here, but also for further studies, such as immunocytochemical analyses of the distribution of Pol in infected cells (D. Knipe, D. Yager, M. Gao, A. Marcy, and D. Coen, unpublished data).

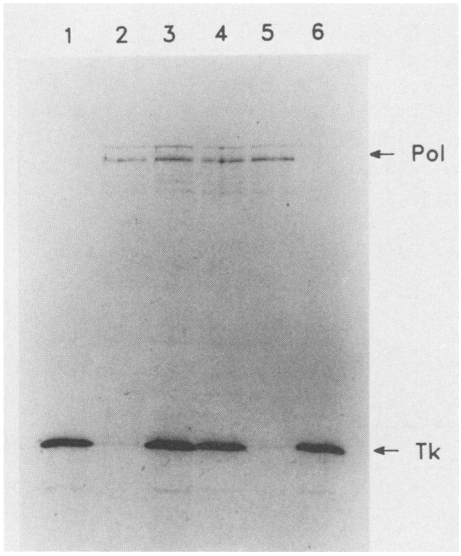


FIG. 6. Comparison of Pol and TK synthesis in cells infected with wild-type HSV and ΔB10. Vero cells were pulse-labeled for 20 min with [$^{35}$S]methionine beginning 6 h after infection with wild-type HSV or ΔB10. Cell lysates were then subjected to three rounds of immunoprecipitation with TK or PP5, or both. Immunoprecipitates were pooled and analyzed by SDS-polyacrylamide gel electrophoresis and quantitated by densitometry of exposed films. Immunoprecipitated proteins from lysates infected with wild-type HSV: lane 1, anti-TK; lane 2, PP5; lane 3, both anti-TK and PP5. Immunoprecipitated proteins from lysates infected with ΔB10: lane 4, anti-TK and PP5; lane 5, PP5; lane 6, anti-TK.

**Pol expression is inefficient at the level of translation.** Comparison of the rates of Pol and TK synthesis demonstrates that the synthesis of Pol is substantially lower at 6 h postinfection than that of TK (Fig. 6), even when differences in transcript levels are taken into account (Table 1). This result, together with our earlier finding that only a minority of *pol* transcripts are present on large polyribosomes (50), leads us to conclude that the initiation of translation on *pol* mRNA is inefficient.

We have previously reviewed features of the *pol* transcript that might influence the efficiency of its translation (50).

TABLE 1. Comparison of Pol synthesis in wild-type- and ΔB10-infected cells

| Virus | Protein | mRNA level[a] (*tk*:*pol*) | Protein synthesis[b] | | |
|---|---|---|---|---|---|
| | | | Units | Normalized value | TK:Pol[c] |
| KOS | TK | 1.2:1 | 1.1 | 1.5 | 6:1 |
| | Pol | | 0.2 | 0.2 | |
| ΔB10 | TK | 1.2:1 | 0.7 | 1.0 | 4:1 |
| | Pol | | 0.2 | 0.2 | |

[a] Transcript levels were determined by quantitative nuclease protection assays, using probes of equivalent specific activity. Nuclease-resistant products were separated by gel electrophoresis and quantitated by scintillation counting.

[b] To determine levels of protein synthesis, pulse-labeled lysates were coimmunoprecipitated and washed and the precipitated material was separated by gel electrophoresis. Exposed films were scanned by using a laser densitometer. Integrated peaks are expressed as arbitrary units and normalized for differences in the number of methionine residues in the two proteins.

[c] Molar ratio of TK and Pol synthesis on a per-transcript basis.

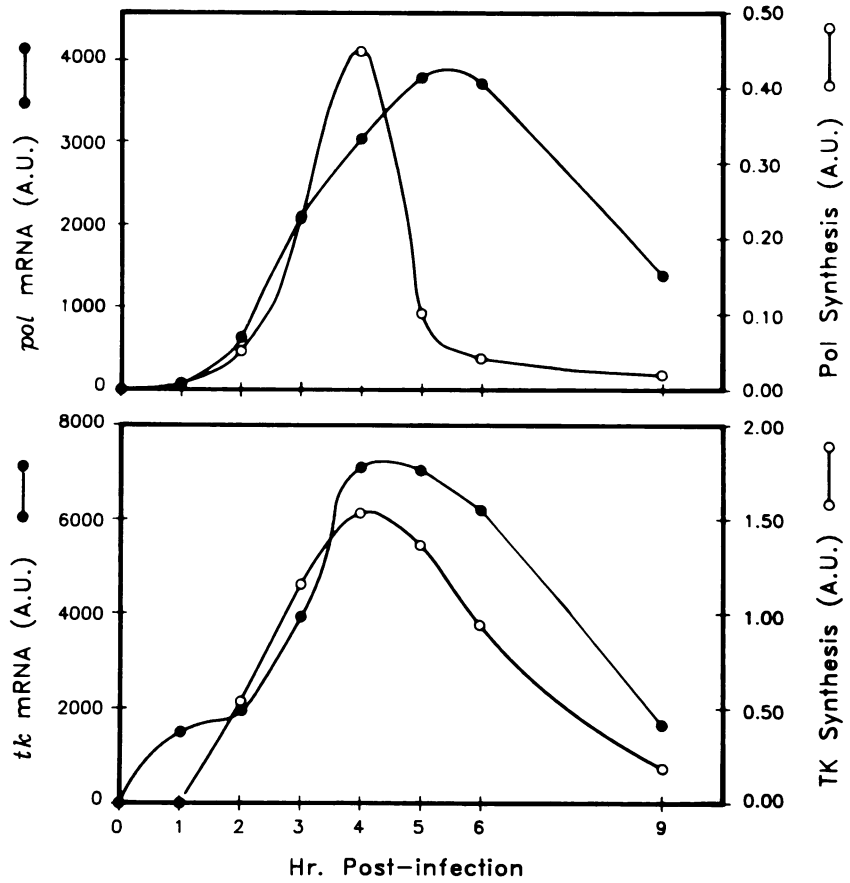


FIG. 7. Temporal regulation of Pol translation. Vero cells were pulse-labeled with [$^{35}$S]methionine for 20 min, and portions were used for measuring protein synthesis or RNA accumulation. Rates of Pol and TK synthesis were determined by immunoprecipitation analysis as for Fig. 8. The results were corrected for differences in methionine content and presented as arbitrary units (A.U.). Transcript levels were determined by measuring the amount of radiolabeled product protected by *tk* and *pol* transcripts from S1 nuclease digestion.

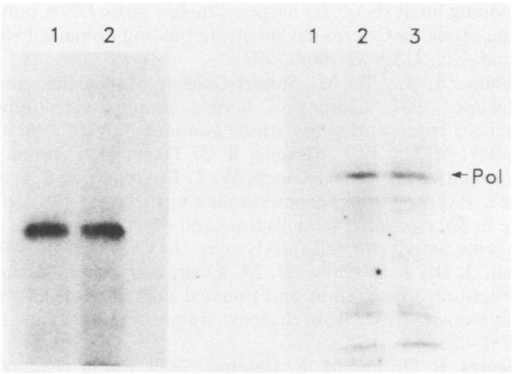


FIG. 8. Analysis of in vitro translation products. Polyadenylated RNA was isolated from cells infected for 2 or 6 h. (Left) Northern blot analysis of poly(A)$^+$) RNA. Samples containing equivalent amounts of *pol* transcripts were fractionated by gel electrophoresis, transferred, and hybridized with a *pol*-specific probe. Lane 1, Poly(A$^+$) RNA isolated at 2 h postinfection; lane 2, poly(A$^+$) RNA isolated at 6 h postinfection. (Right) Immunoprecipitation of Pol synthesized in rabbit reticulocyte lysates. Lane 1, Protein synthesized in lysates programmed with no RNA; lanes 2 and 3, protein synthesized from RNA isolated at 2 and at 6 h postinfection, respectively.

These features include the context of the initiation codon of the major *pol* ORF, which differs from the optimal consensus sequence (19), a short ORF upstream of the major *pol* ORF, and the potential for secondary structure. In the work presented here, the removal of sequences from positions +57 to +199, which include the upstream ORF, had only a slight effect on Pol translation (Fig. 6, Table 1). This finding contrasts with studies of translation of uncapped synthetic *pol* transcripts in rabbit reticulocyte lysates in which transcripts with 5′ termini at position +129 (downstream of the short ORF) were reported to be translated more efficiently than transcripts with 5′ termini at position +57 (7). This observed difference could be due to many factors, including differences in mRNA caps (i.e., uncapped transcripts were used in the in vitro studies), 5′ ends, and translational mechanisms in cell extracts relative to the in vivo conditions used in our studies.

A 144-nucleotide hairpin structure encompassing the Pol initiation codon has been previously predicted (14). This structure would have a Gibbs free energy of −71 kcal/mol (ca. −300 kJ/mol). Such a hairpin would be more stable than those shown to impair strongly the translation of preproinsulin, *tk*, and human immunodeficiency virus type 1 transcripts (21, 34, 35, 41). The deletion in mutant ΔB10 removes 32 nucleotides of this predicted hairpin, with little discernible effect on Pol expression. We cannot exclude the possi-

bility that the sequences remaining after the deletion still form a hairpin stable enough to impair Pol translation. Alternatively, modeling using the program PCFOLD (52) predicts that *pol* transcripts made in wild-type or ΔB10 infections could form structures involving the first 55 nucleotides and sequences immediately downstream from the Pol AUG initiation codon. This structure would have a Gibbs free energy of −112 kcal/mol (ca. 469 kJ/mol) (results not shown). Such a structure might prevent access of initiation factors to the 5′ cap (30, 35, 36). Preliminary experiments reveal an increased expression of Pol from transcripts without the first 55 nucleotides in rabbit reticulocyte lysates and in transient expression assays of transfected cells (results not shown). Further mutational analysis of these regions will be required to identify elements important for the inefficient translation of Pol.

**Temporal regulation of Pol.** A question that remains to be addressed is why does this virus regulate *pol* expression at the level of translation? We previously suggested two possible reasons (50). The first of these was that most delayed-early genes might be transcribed at similar rates, with the result that posttranscriptional mechanisms would be required to control stoichiometries of viral replication proteins. However, a report that delayed-early viral genes have widely divergent transcription rates (51) makes this hypothesis less tenable. A second possibility is that translational control might afford HSV finer temporal control of Pol expression.

The findings reported here support the second hypothesis. The rate of Pol synthesis peaked by 4 h postinfection (Fig. 5A and 7) while accumulation of *pol* transcripts continued to increase for another 2 h (Fig. 7) (46, 50). At present, on the basis of the results from in vitro translation, there is no indication that *pol* transcripts are altered during the course of infection. The apparent decline in the translation efficiency of *pol* mRNA during infection may be due to its relative inability to compete for limiting initiation factors (25). These factors could become limiting because of increasing levels of late transcripts accumulating in the infected-cell cytoplasm or because shutoff of host cell expression induced by HSV infection prevents replenishment of one or more of these factors. Regardless of whether this or other mechanisms are responsible for the decline of Pol expression, it is clear that Pol synthesis shuts off more quickly than at least one other delayed-early gene. This may be advantageous to the virus by preventing counterproductive interactions of Pol with other replication proteins.

One remaining hypothesis involves the possibility of a functional relationship between *pol* transcription and the initiation of DNA synthesis from the adjacent origin of replication, $ori_L$. Transcription factors and/or transcription per se is required for origin activity in several viral and cellular systems (reviewed in references 1, 5, and 32). A high level of *pol* transcription might be necessary for, or the result of, the activation of DNA replication from $ori_L$. As a consequence, posttranscriptional regulation (e.g., at the level of translation) would be required in order to prevent overexpression of Pol. Such a mechanism has been suggested for the control of baculovirus DNA polymerase expression (44). The finding that mutants lacking a functional $ori_L$ are viable (37) should facilitate testing of this hypothesis.

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