JOURNAL OF VIROLOGY, May 1995, p. 2811–2818 Vol. 69, No. 5
0022-538X/95/$04.00+0

Cloning, Sequencing, and Functional Characterization of the Two Subunits of the Pseudorabies Virus DNA Polymerase Holoenzyme: Evidence for Specificity of Interaction

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Received 15 July 1994/Accepted 27 January 1995

The pseudorabies virus (PRV) genes encoding the two subunits of the DNA polymerase were located on the genome by hybridization to their herpes simplex virus type 1 (HSV-1) homologs, *pol* and UL42, and subsequently were sequenced. Like the HSV-1 homologs, in vitro translation products of the PRV gene encoding the catalytic subunit (*pol*) possessed activity in the absence of the Pol accessory protein (PAP). However, the PRV PAP stimulated the activity of Pol fourfold in the presence of 150 mM KCl, using an activated calf thymus DNA template. The stimulation of Pol activity by PAP under high-salt conditions and the inhibition of Pol activity by PAP when assayed in low salt (0 mM KCl) together were used to determine the specificity with which PAP interacted with Pol. Despite functional similarity, HSV-1 UL42 and PRV PAP could neither stimulate the noncognate Pols at high salt nor inhibit them at low salt. Furthermore, a PRV Pol mutant lacking the 30 C-terminal amino acids retained basal Pol activity but could be neither stimulated nor inhibited by the PRV PAP. Sequence comparisons of the Pol proteins of the alphaherpesviruses reveal a conserved domain in the C terminus which terminates immediately before the last 41 residues of both PRV and HSV-1 proteins. These results indicate that the ability and specificity for interaction of the PRV Pol with PAP most likely resides predominantly in the extreme Pol C terminus.

DNA replication is a complex phenomenon that has been studied in a variety of prokaryotic and eukaryotic organisms and viruses. Herpesviruses encode most of the enzymes directly involved in DNA replication (8, 73) and therefore provide attractive models for understanding the mechanism of this process. In particular, they have proven to be good models for determining the interactions among the various components of the replication apparatus (10, 16, 23, 29, 35, 67). Moreover, the components of the herpesvirus DNA replication complex and the specific interactions that they share are of interest as targets for the development of antiviral chemotherapy.

Pseudorabies virus (PRV) and herpes simplex virus type 1 (HSV-1) are members of the alphaherpesvirus subfamily and exhibit many common characteristics, including the ability to replicate in epithelial tissues and to establish latency in the neural tissues of swine and humans, respectively (60). These viruses are quite similar in genomic structure and organization, although an inversion, from approximately 0.1 to 0.4 map unit in the UL region of the genome of PRV (4, 12), and a contraction of the US region, compared with HSV-1, have been reported (66). However, the G+C contents of the genomes differ considerably, and cross-hybridization experiments indicate that the genomes share a maximum of 8% nucleotide sequence homology (6, 58). While some aspects of PRV genome replication have been studied (3, 44), the essential DNA replication functions have not been identified. Identification of the components of the PRV DNA replication complex and analysis of the protein-protein interactions which they form would lend greater insight into the function, specificity, and importance of particular interactions.

Two of the seven HSV-1 proteins which are required in *trans* for the amplification of plasmids containing an HSV-1 origin of replication (73) form a heterodimeric complex which appears to function as the DNA polymerase holoenzyme (9, 29, 35). The catalytic subunit of the DNA polymerase gene (*pol*) encodes a 1,235-amino-acid protein with an apparent molecular weight of 140,000 (27, 50, 57). This polypeptide possesses inherent enzymatic properties including DNA chain elongation (17, 30, 48), 3′-5′ exonuclease (41, 48), and RNase H (9, 48) activities. It has been shown to be tightly associated with a 65-kDa double-stranded DNA-binding protein encoded by the UL42 gene of HSV-1 (9, 23, 29, 35, 55, 68), a gene that is essential for DNA synthesis and virus replication (38, 47). The product of the UL42 gene stimulates the basal activity and processivity of Pol (22, 29, 35), properties which appear to be essential for HSV-1 replication (13, 14, 59, 63).

We were interested in identifying the PRV counterparts of this enzyme complex in an effort to examine the specificity of their interaction. Homologs for both Pol and UL42 have been identified for a variety of herpesviruses (1, 2, 11, 19, 40, 45, 51, 64), and for the homologs of human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV), functional interaction between the two proteins was demonstrated (19, 40). However, examination of cross-species interaction of the components among members of the herpesvirus family has not been published.

In this report, we describe the identification, cloning, sequencing, and functional characterization of the PRV Pol and

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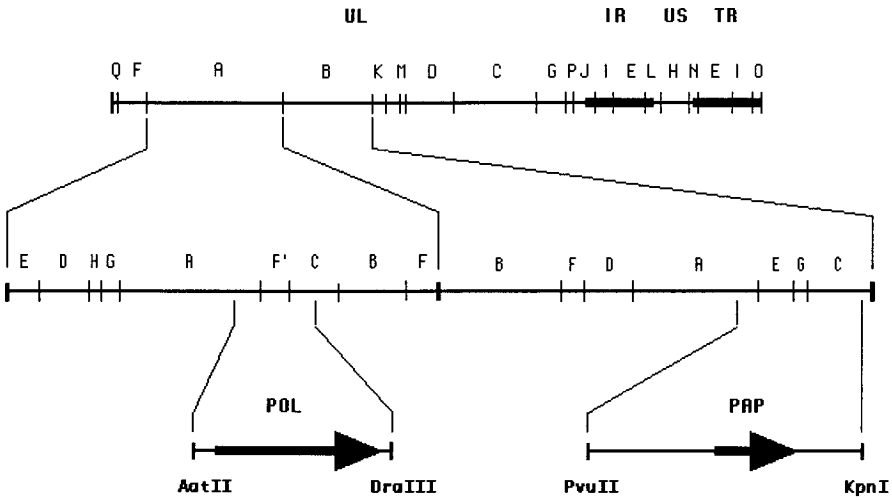

FIG. 1. Localization of the PRV *pol* and *pap* genes. The upper line shows the *Bam*HI restriction endonuclease map of the PRV genome (52, 71). Solid boxes represent the inverted repeats that bracket the short unique region of the genome. The second line corresponds to an expanded representation of the *Bam*HI A and B fragments. *Sal*I subfragments of *Bam*HI-A and *Nru*I subfragments of *Bam*HI-B are indicated above the line. Positions of the *pol* and *pap* genes are shown at the bottom; their orientations are indicated by arrows.

UL42 homologs. Using in vitro transcription-translation products, we demonstrate that the PRV UL42 homolog stimulates the activity of the PRV Pol and that the C-terminal 30 residues of the PRV Pol are not essential for basal activity but are required for stimulation by the Pol accessory protein (PAP). Stimulation of Pol activity did not occur with heterologous mixes between HSV and PRV proteins. Furthermore, we show that the salt optimum for activity of the PRV Pol catalytic subunit is substantially different from that for the PRV holoenzyme, and this property was used to confirm the lack of functional association of heterologous components. We also have compared Pol and PAP sequences among different herpesviruses and provide additional insight into the importance of various domains of these proteins.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (Vero) were obtained from the American Type Culture Collection (Rockville, Md.). Cells were propagated in Eagle's minimum essential medium supplemented with 5% heat-inactivated fetal bovine serum in a 37°C incubator with a 5% CO_2 atmosphere. HSV-1 strain F (33) and PRV strain Ka (39) were obtained from B. Roizman and T. Ben-Porat, respectively. Both virus strains were propagated in Vero cells at low input multiplicity of infection (0.01 PFU per cell). Virus yields were determined by plaque assay on Vero cell monolayers at 34°C in 5% CO_2. For the production of PRV and HSV-1 DNA, Vero cells were infected at a multiplicity of infection of 5 PFU per cell.

Hybridization analysis. Virus DNA was purified from the cytoplasm of infected cells by banding in NaI equilibrium density gradients by the method of Walboomers and Ter Scheggett (69). After size fractionation of restriction endonuclease-treated DNA by agarose gel electrophoresis, the DNA was denatured before being transferred to Hybond N nylon filters (Amersham). Dried and baked filters were prehybridized at 45°C for 24 h in buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll), 1% sodium dodecyl sulfate (SDS), 100 μg of denatured herring sperm DNA per ml, and 40% deionized formamide.

Probes for hybridization were prepared by isolating, from agarose gels, fragments of HSV-1 DNA that had been subcloned into pSK^+ vectors, and the purified DNA was labelled with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; Amersham) by the random priming method (20), using a kit (Amersham) as instructed by the manufacturer. Approximately 2×10^6 cpm of labelled denatured probe was applied to each filter. Hybridization conditions were the same as above except that the concentration of Denhardt's solution was reduced 10-fold. Filters were washed at 45°C in 6× SSC–0.1% SDS, dried, and exposed to X-ray film at −70°C, using an intensifying screen.

Cloning procedure. For cloning of the *pol* gene, a plasmid containing the 29.4-kbp *Bam*HI A fragment of PRV DNA (kindly provided by H. J. Rziha, Tubingen, Germany) was digested with restriction enzyme *Aat*II and was partially cleaved by enzyme *Dra*III to yield an *Aat*II-*Dra*III subfragment 3.4 kbp in size (Fig. 1). After removal of the 3′ overhangs with the Klenow enzyme, this fragment was cloned into the *Eco*RV site of the polylinker of the pBluescript SK^+ vector (Stratagene) to form plasmid pPRVpol. This plasmid contains the PRV *pol* open reading frame (ORF) as well as 133 bp upstream from the putative translation initiation codon and 142 bp downstream from the consensus polyadenylation signal. Cloning of the PRV PAP was initiated with a plasmid containing the 18.4-kbp *Bam*HI B fragment of PRV DNA (gift of H. J. Rziha). The 5.9-kbp *Pvu*II-*Kpn*I subfragment (Fig. 1) was cloned into the *Eco*RV and *Kpn*I sites of pBluescript SK^+ to form plasmid pPRVpk5.9. This plasmid was linearized at the *Kpn*I site, the 3′ overhangs were removed with the Klenow enzyme, the DNA was partially digested with *Nru*I, and the mixture was religated, generating plasmid pPRVpn3.2. Unidirectional progressive deletion from the *Pvu*II extremity of pPRVpn3.2 was performed by the exonuclease III method of Henikoff (34), generating plasmid pPRVpap. This plasmid contains the PRV *pap* ORF as well as 15 bp upstream from the putative translation initiation codon and 53 bp downstream from the consensus polyadenylation signal. Plasmids pLBN 19A, encoding full-length HSV-1 UL42 (22), and pT7-7.1, encoding HSV-1 Pol lacking the first 67 amino acids (17), have been described elsewhere.

DNA sequencing. Nested sets of progressive unidirectional deletions from both extremities were prepared from plasmids pPRVpol and pPRVpn3.2 (34). Selected clones were amplified in *Escherichia coli*, and DNA was isolated by an alkaline lysis procedure (62). All plasmids were sequenced by the dideoxynucleotide chain termination method (61), using the DNA polymerase T7 sequencing kit (Pharmacia) and [α-^{35}S]dATP (Amersham). To prevent secondary structures in DNA during sequencing, 10% dimethyl sulfoxide was added to the reaction mixtures. Deaza C-7 dGTP or dITP was used to resolve band compressions due to the high G+C nucleotide content of the PRV DNA. Both strands of DNA from each plasmid were sequenced at least two times, using progressive deletions. The sequences were further confirmed by subcloning and sequencing smaller restriction fragments from the parental plasmid or by direct sequencing using selected synthetic oligonucleotides as primers. DNA sequences were analyzed for eukaryotic transcriptional elements by using the SIGNAL SCAN software (56).

In vitro transcription-translation. Ten micrograms of pPRVpol, pPRVpap, pT7-7.1 (HSV-1 *pol*), and pLBN 19A (HSV-1 UL42) were linearized downstream of each ORF with *Cla*I, *Pvu*II, *Xba*I, and *Xba*I, respectively. The linearized DNA was purified by phenol-chloroform extraction and concentrated by ethanol precipitation. In vitro transcripts were generated with a kit (Promega Biotec, Madison, Wis.) as instructed by the manufacturer. Briefly, 3 μg of purified DNA was transcribed with 15 U/μg of DNA (for pPRVpap and pLBN 19A) or 10 U/μg of DNA (for pPRVpol and pT7-7.1), using T3 or T7 RNA polymerase, as appropriate, in the transcription buffer provided, with 10 mM dithiothreitol, 1.6 U of RNasin RNase inhibitor (Promega Biotec) per μl, 0.5 mM each ribonucleoside triphosphate, and diethyl pyrocarbonate-treated water. After 1 h of incubation at 40°C, linearized DNA was removed with RQ1 RNase-free DNase (1 U/μg of DNA; Promega Biotec) for 15 min at 37°C. The RNA was purified by phenol-chloroform extraction and ethanol precipitation and analyzed on formaldehyde-agarose gels as described previously (46).

Transcripts were translated in rabbit reticulocyte lysates as directed by the manufacturer (Promega Biotec). For each reaction, 0.5 to 1 μg of RNA was incubated for 1 h at 30°C with 0.8 μCi of L-[^{35}S]methionine (specific activity, 1,000 to 1,300 Ci/mmol; Amersham) per μl. Products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using 10 to 20% polyacrylamide gradient gels, impregnated with En^{3}Hance (DuPont, Boston, Mass.), and exposed to X-ray film. For Pol enzyme assays, 5 to 10 μg of RNA was translated in the presence of unlabelled L-methionine. In some cases, a coupled transcription-translation system (Promega Biotec) was used to generate proteins.

Pol assay. Pol activity was measured as incorporation of [^{3}H]dTTP (42 Ci/mmol; Amersham) into trichloroacetic acid-insoluble radioactivity as described previously, using activated calf thymus DNA as the template (17, 18, 22), except that KCl concentration was varied as indicated in the figure legends. In vitro translation products from the indicated *pol* and *pap* genes (25 μl) were added and incubated in a final volume of 100 μl for 30 min at 37°C. For reactions containing both Pol and PAP, the translated products were preincubated 5 min at 37°C before initiation of the reaction in order to allow complex formation. All assays were conducted in duplicate. Units of Pol activity are defined as the number of femtomoles of [^{3}H]dTTP incorporated into DNA at 37°C during 30 min by 25 μl of programmed lysate.

Nucleotide sequence accession numbers. The nucleotide sequences corresponding to the PRV Pol and PAP ORFs have been submitted to the GenBank database and have been assigned accession numbers L24487 and M94355, respectively.

RESULTS

Localization and sequencing of the PRV *pol* gene. Heterologous hybridization experiments were performed by probing a Southern blot containing *Bam*HI-restricted PRV genomic DNA (strain Ka) with a subcloned 3.4-kbp *Bam*HI R restriction fragment of the HSV-1 (strain F) genome. The HSV-1 fragment was separated from plasmid DNA by gel electrophoresis before use as a probe for hybridization. This probe contained 154 nucleotides upstream from the initiation codon to nucleotide residue 3216 of the HSV-1 *pol* ORF. Positive specific hybridization was observed with the 29.4-kbp *Bam*HI A PRV DNA fragment (Fig. 1), suggesting that PRV *pol* was included within this region. Further experiments using the same probe and PRV *Bam*HI-A DNA cleaved with *Sal*I revealed that the HSV-1 *pol* gene hybridized with the PRV *Sal*I A, F′, and C subfragments (52). Preliminary DNA sequencing revealed *Aat*II and *Dra*III sites, respectively, upstream and downstream from the PRV *pol* ORF. Complete sequencing of the *Aat*II-*Dra*III subfragment confirmed that it contained the entire PRV *pol* gene (Fig. 1). Analysis of the PRV *pol* nucleotide sequence reveals that the gene contains a 3,144-bp continuous ORF, predicted to encode a protein of 1,048 amino acids. At the 3′ end of the PRV *pol* ORF, there is a canonical polyadenylation signal which is 15 nucleotides downstream of the termination codon UGA. The first AUG codon, presumed to be the start site for translation, is found in region GAGC GAUGG, in good agreement with the requirements of active translation initiation site (43), with a guanine at position +4 and a purine at position −3. That this is the true initiation codon is further suggested by the lack of another methionine codon until codon 425 and by the fact that the N-terminal portion of the PRV *pol* ORF just downstream from the putative AUG initiation codon displays conservation with that of other alphaherpesviruses (data not shown). Using the SIGNAL SCAN computer program that scans DNA sequences for eukaryotic transcription elements, we detected several consensus regulatory sequences, including a consensus TATA box at nucleotide −44 and several consensus Sp1 sites between nucleotides −57 and −110, in the region upstream of the *pol* ORF. In contrast to the genomic features in the region of the HSV-1 *pol*, neither a small ORF nor sequences resembling a replication origin (27, 50, 57) have been found near the PRV *pol* promoter.

Localization and sequencing of the PRV *pap* gene. To locate the PRV sequences corresponding to the HSV-1 PAP, UL42, a Southern blot of *Bam*HI-digested PRV genomic DNA was probed with a cloned 1.3-kbp *Mlu*I fragment, encompassing sequences located entirely within the HSV-1 (strain F) UL42 ORF. Specific hybridization of the probe to the 18.4-kbp PRV *Bam*HI B fragment provided a rough localization of the PRV UL42 homolog, *pap* (Fig. 1). To further define the location of the gene, a plasmid containing the PRV *Bam*HI B fragment was digested in a series of experiments with *Kpn*I, *Pvu*II, and/or *Nru*I, Southern blots of separated fragments were prepared, and the blots were hybridized to an HSV-1 *Mlu*I UL42 probe.

Complete sequencing of the *Nru* E and G subfragments indicated that they contained the 5′ extremity of the *vhs* gene (5) and the entire putative PRV *pap* gene. Analysis of the *pap* nucleotide sequence reveals that the gene contains a 1,152-bp continuous ORF encoding 384 amino acids. Both the first and second AUG codons in the ORF are found in regions which are in relatively good agreement with Kozak's rules for translation initiation (43). However, on the basis of conservation of the upstream residues with those of other alphaherpesvirus homologs, it seems more likely that the protein initiates at the upstream AUG codon. Several putative regulatory sequences, including a degenerate TATA box homology (TAAAATA) located at −100, three overlapping Sp1 consensus elements located between −86 and −76, and a perfect TATA box homology (ATATAA) at −63, were detected upstream from this presumed initiation codon. Interestingly, at the 3′ end of the *pap* ORF is a canonical polyadenylation signal (AATAAA) which overlaps the termination codon TAA. The relative orientations of the PRV *pol* and *pap* genes are as in the HSV-1 prototype orientation (50).

Characterization of PRV *pol* and *pap* translation products. Runoff transcripts were produced by transcription in vitro with the T3 RNA polymerase of plasmids pPRVpol and pPRVpap linearized downstream of the ORFs. These RNAs were translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and analyzed by SDS-PAGE. The PRV gene products were compared with those translated from RNA generated by the T7 RNA polymerase runoff transcription of the linearized plasmid pT7-7.1, coding for the HSV-1 Pol which lacks the 67 amino-terminal amino acids, and pLBN 19A, encoding the HSV-1 UL42 gene (Fig. 2). The major translation product of the PRV *pol* gene corresponded to a protein with an apparent molecular weight of 110,000 (lane 2), whereas the major translation product produced from the HSV-1 gene migrated as a 130-kDa peptide (lane 4). The major translation product of the HSV-1 UL42 gene has an apparent molecular mass of 62 kDa (lane 5) as previously described (55), whereas that of the PRV homolog is considerably smaller, migrating as a 42-kDa polypeptide (lane 3). The sizes of both the PRV *pol* and *pap* gene products are in good agreement with the 115.3 and 40.3 kDa predicted from the ORFs.

Biological activities of PRV Pol and PAP. To determine whether the PRV Pol and PAP homologs were able to function as predicted, we performed DNA polymerase assays on in vitro transcription-translation products of the genes. Table 1 demonstrates that like the HSV-1 Pol, the PRV Pol possesses activity on activated calf thymus DNA template at 150 mM KCl even in the absence of the accessory protein. We observed no significant Pol activity either in unprogrammed rabbit reticulocytes or in translation products of either the PRV *pap* or the HSV-1 UL42 gene. However, when we mixed the PRV *pap* and PRV *pol* translation products, we observed a 3.5-fold stimulation of Pol activity compared with the activity of Pol products alone. In parallel assays, we observed a 3.4-fold stimulation of HSV-1 Pol activity in the presence of the HSV-1 UL42. These results demonstrate that the PRV gene that we have

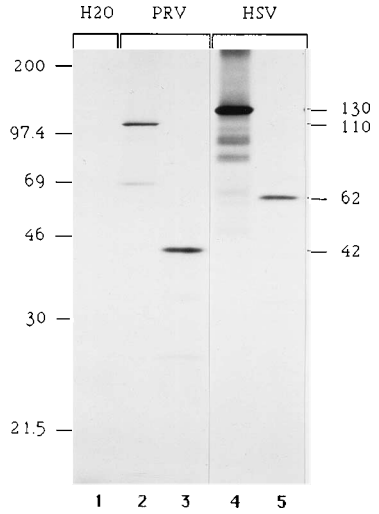

FIG. 2. In vitro translation products of the PRV and HSV-1 *pol* and *pap* genes. RNA transcribed by in vitro runoff transcription from linearized DNA templates (pPRVpol, pPRVpap, pT7-7.1 [HSV-1 *pol*]) and pLBN 19A [HSV-1 UL42]) were translated in rabbit reticulocyte lysate with ^{35}S-labelled L-methionine for 1 h at 30°C. Products were separated through a denaturing 10 to 20% gradient polyacrylamide gel. Rabbit reticulocyte lysates were programmed with water (lane 1), PRV *pol* (lane 2), PRV *pap* (lane 3), HSV-1 *pol* (lane 4), and HSV-1 UL42 (lane 5) RNAs. The molecular masses (in kilodaltons) of protein standards are indicated on the left; the apparent masses of the full-length translation products are shown on the right.

designated *pap* encodes a Pol accessory protein and possesses activity similar to that of its HSV-1 counterpart.

Effect of salt concentration on Pol activity. Because the HSV-1 Pol holoenzyme has been shown to be stimulated in the presence of high salt, we investigated the sensitivity of the PRV Pol or Pol-PAP complex to salt. In vitro transcription-translation products of the PRV and HSV-1 genes were assayed for Pol activity in the presence of increasing concentrations of KCl. As previously observed for the HSV-1 Pol catalytic subunit (32), we found the activity of the PRV Pol to be extremely sensitive to salt in the absence of its accessory protein, with maximum activity observed when no exogenous KCl was added (data not shown). Indeed, in the absence of exogenously added KCl, the PRV Pol exhibited more than eight times the activity found in the presence of 150 mM KCl (compare bar 1 in Fig. 3B with bar 1 in Fig. 3A). Although the in vitro-translated HSV-1 Pol activity was somewhat lower than that of the PRV Pol, we observed a similar level of decrease in activity when the exogenous KCl concentration was raised from 0 to 150 mM KCl (compare bar 6 in Fig. 3B with bar 6 in Fig. 3A). The opposite effect of salt on activity was observed for both the PRV and HSV-1 Pols in the presence of their respective accessory proteins: the activity of each Pol-PAP complex was higher in the presence of 150 mM KCl than in the presence of 0 mM KCl (compare bars 2 and 7 in Fig. 3A with bars 2 and 7 in Fig. 3B). The net effect of these interactions is that the accessory proteins inhibit the activities of the Pols in low salt and stimulate their activities in the presence of high salt (Fig. 3; compare bars 2 and 1 and bars 7 and 6). Although the latter effect has been one of several means for ascribing Pol accessory function to HSV-1 UL42 and some of its homologs (14, 19, 22, 23, 29, 35, 40, 65), it is of particular note that the maximum activities that we observed for the HSV-1 and PRV Pol-PAP complexes (in high salt) never exceeded the maximum activities of the Pols without their accessory proteins (in low salt) (Fig. 3 and results not shown).

We took advantage of the dramatic differences in salt sensitivities of the Pol-PAP complexes compared with those of the Pol catalytic subunits alone to investigate the specificity of the PAPs. When we mixed the heterologous HSV-1 UL42 with the PRV Pol, we found no effect on PRV Pol activity at either 0 or

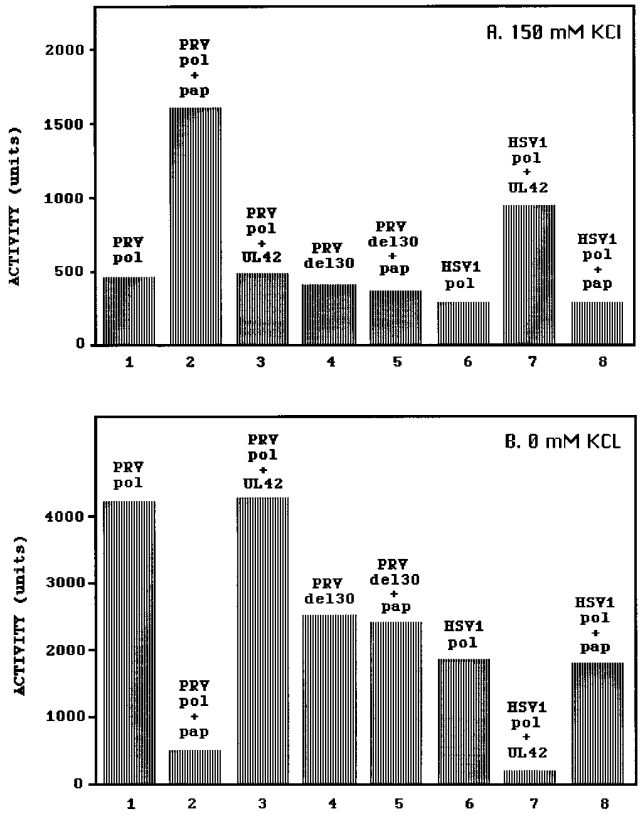

FIG. 3. Specificity of interaction between Pols and PAPs from PRV and HSV-1 and lack of activation of PRV Pol del30 by its cognate cofactor. Pol activity was assayed for nonlabelled translation products from PRV *pol* and *pap*, PRV *pol* del30, and HSV-1 *pol* and UL42, with an activated calf thymus DNA template, in the presence of 150 (A) or 0 (B) mM KCl. Bars indicate averages of duplicate experiments. Neither the PRV PAP nor the HSV-1 UL42 protein possessed activity in excess of that for the unprogrammed lysates, which accounted for less than 10% of the observed incorporation rates.

TABLE 1. Activities of PAPs on wild-type Pol proteins

Construct	Pol activity (U)[a]	Fold stimulation[b]
PAP	0	0.0
PRVpol (wt[c])	470 ± 49	1.0
PRVpol (wt) + PAP	1,620 ± 115	3.5
UL42	0	0.0
HSVpol	360 ± 31	1.0
HSVpol + UL42	1,210 ± 72	3.4

[a] Determined by assay of products from coupled in vitro transcription-translation reactions on activated calf thymus DNA template in the presence of 150 mM KCl as described in Materials and Methods. Pol assays were performed as duplicates for four independent experiments, and the means and standard deviations are shown. Activities were calculated by subtracting the radioactivity incorporated by unprogrammed rabbit reticulocyte lysates from the values obtained with each RNA indicated. Incorporation by lysates programmed with PAP or UL42 were indistinguishable from those from unprogrammed lysates, which never exceed 10% of the level incorporated by the Pols alone.

[b] Calculated as the mean activity of the indicated Pol in the presence of cofactor divided by the mean activity of that Pol in the absence of cofactor.

[c] wt, wild type.

150 mM KCl (Fig. 3; compare bar 3 with bar 1). Likewise, a heterologous mix of PRV PAP with HSV-1 Pol yielded neither an increase in activity of Pol at 150 mM KCl nor a decrease in activity at 0 mM KCl (Fig. 3; compare bar 8 with bar 6). Taken together, these results extend those of Hart and Boehme (32) in demonstrating the specificity of both the stimulation of Pol activity in high salt and the inhibition of Pol activity in low salt by PAPs. Furthermore, they suggest that cross-species Pol-PAP complex formation does not occur among the HSV-1 and PRV components.

Consistent with the interpretation that these effects are the results of specific complex formation between Pol and PAP are results obtained for one PRV Pol deletion mutant. PRV Pol deleted of the C-terminal 30 amino acids (del30) was generated by cleavage of pPRVpol DNA with *Stu*I prior to runoff transcription followed by in vitro translation. PRV del30 possessed activity similar to that of full-length PRV Pol at 150 mM KCl in the absence of accessory protein (Fig. 3A; compare bar 4 with bar 1), yet the addition of PRV PAP failed to enhance its activity (compare bars 4 and 5). Although the activity of the PRV Pol del30 was somewhat lower than that of the full-length Pol at 0 mM KCl, the addition of PRV PAP was not inhibitory (Fig. 3B; compare bars 4 and 5 with bars 1 and 2). These results demonstrate that the C-terminal 30 residues of PRV Pol are not necessary for basal activity but are required for functional interaction with PAP.

DISCUSSION

Comparison of PRV Pol sequences with sequences of herpesvirus homologs. Using the CLUSTALV computer program (36), we have aligned the PRV Pol and PAP sequences with those of HSV-1 (50), varicella-zoster virus (VZV) (11), and equine herpesvirus 1 (EHV-1) (64), all members of the alphaherpesvirus family. In addition, we have included the EBV, HCMV, and human herpesvirus 6 (HHV-6) putative homologs, obtained from the SwissProt database. Employing pairwise alignments, the PRV *pol*, *pap*, and previously reported *vhs* (5) genes are most closely related to those of EHV-1, in agreement with the placement of EHV-1 and PRV on the same branch of an evolutionary tree (49).

In addition to the eight regions of homology (I through VII and A) observed among a variety of prokaryotic, eukaryotic, and viral α-like DNA polymerases (26, 37, 70, 72), there are several additional regions of sequence homology among the herpesvirus *pol* genes. Among these, a C-terminal domain, extending from PRV residues 949 to 1007, is well conserved between the alphaherpesvirus group and is somewhat more divergent when EBV, HCMV, and HHV-6 are included in the alignment (Fig. 4A). This domain is included in the HSV-1 Pol region described by Digard and Coen (15) to be necessary and sufficient for stable association with the UL42 PAP. However, within the last 41 residues of both the PRV and HSV-1 *pol* genes are only three residues identical to those of the other alphaherpesvirus homologs, and only one if we include the other three viruses. Interestingly, deletion of the C-terminal 30 residues of PRV Pol abolishes its ability to be stimulated by its cognate accessory protein, while having little or no effect on basal Pol activity (Fig. 3). The C terminus of HSV-1 Pol also has been shown to be required for stimulation by UL42 but not for basal polymerase activity (13, 63, 65). The HSV-1 Pol was shown to possess catalytic activity even after deletion of residues 1073 to 1144 or of the 59 C-terminal amino acids (30). Several other groups have confirmed these results and have shown that the HSV-1 Pol C terminus appears to be critical for functional interaction with UL42, although the exact delineation of the critical residues remains uncertain (13, 63, 65). Of particular note, however, is the fact that an HSV-1 Pol mutant lacking the C-terminal 27 residues and expressed as a baculovirus recombinant possessed activity which could not be stimulated by UL42 in vitro and which could not function in origin-dependent DNA replication (13, 63). Thus, it appears clear that the residues which lie downstream of the conserved C-terminal domain are essential for true functional interaction

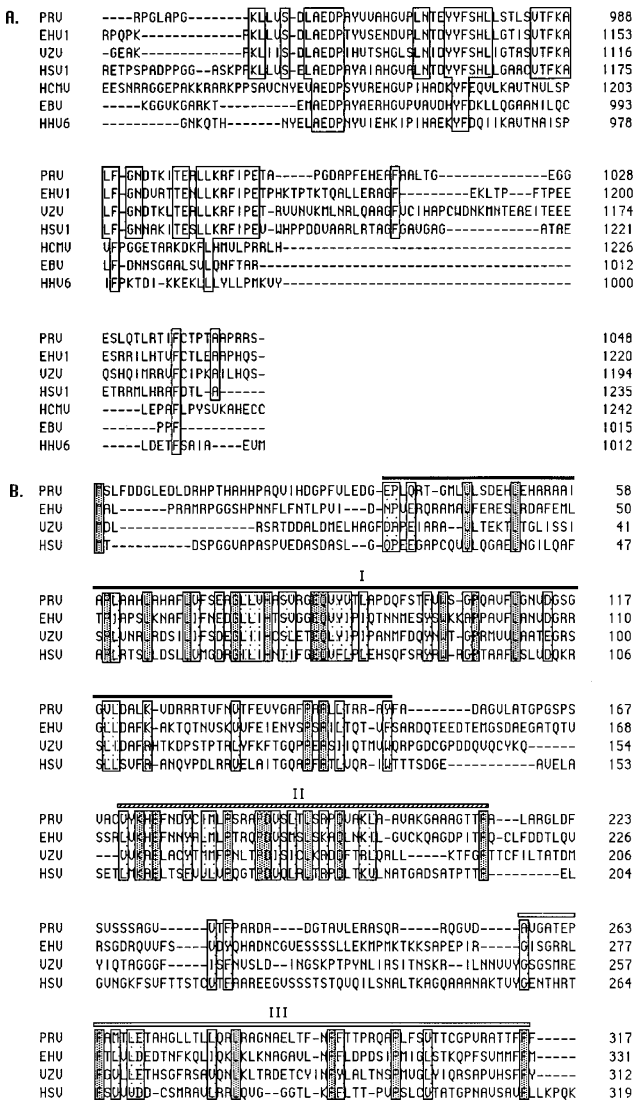

FIG. 4. (A) Alignment of the predicted amino acid sequence of the C-terminal domain of the PRV *pol* gene product with counterparts from HSV-1, VZV, EHV-1, HCMV, HHV-6, and EBV. Sequences were aligned by using the multiple alignment CLUSTALV computer program (36). Open boxes indicate the positions of identical amino acids either in the four alphaherpesviruses or in the seven sequences. Numbers at the right indicate amino acid numbers. (B) Alignment of three conserved domains of the PAPs from four alphaherpesviruses. The PRV PAP was aligned with its counterparts from HSV-1 (UL42 gene), VZV (gene 16), and EHV-1 (gene 18). Sequences were aligned by using the multiple alignment CLUSTALV computer program. The limits of the three domains were defined using arbitrary criteria (5 identical or conserved residues in 25). The positions of identical amino acids in all four sequences are indicated with darkly shaded boxes, while those of conserved amino acids are shown as lightly shaded boxes. The following residues were considered to be conserved: MILCV, DEQN, PAG, FYW, KRH, and ST. The limits of conserved domains I (solid bar), II (hatched bar), and III (open bar) are represented above the sequences. Numbers at the right indicate amino acid numbers.

with their respective accessory proteins. The high level of divergence of these sequences may, in fact, reflect the specificity of interaction with the accessory proteins which are highly divergent among the herpesviruses. It is interesting, however, that the divergent C-terminal Pol region is much shorter in HCMV and HHV-6 and is almost completely absent in EBV, with the notable exception of one phenylalanine residue which is conserved in all the seven polymerases (Fig. 4A). Site-directed mutagenesis of this region of Pol will be necessary to determine those residues which are most critical for interaction with these proteins.

Comparison of PAP sequences. We attempted to align PAP sequences from the seven herpesviruses. Significant sequence conservation was found within the alpha- and the betaherpesvirus groups, but little conservation was found between them (not shown). Comparison of the UL42 homologs of the four alphaherpesviruses is consistent with the three-way alignment previously reported for HSV-1, EHV-1, and VZV homologs (65) and shows that the amino-terminal two-thirds to three-fourths of the protein is best conserved (Fig. 4B). In the conserved portion of PAP, we have distinguished three distinct conserved domains. Domain I corresponds to PRV amino acids E-36 to Y-152, domain II corresponds to residues V-171 to F-215, and domain III corresponds to residues A-257 to F-316. Although these three domains correspond to only 57% of the PRV polypeptide, they include all of the identical amino acids and 94% of the similar residues. Although the relationship between the structure and the function of the HSV-1 UL42 protein is not completely understood, most of the results from mutagenesis studies support the importance of the conserved domains in the amino-terminal portion of the HSV-1 UL42 (14, 53, 59). The strongest divergence, which occurs downstream of PRV residue 317, results not only from the longer HSV-1 C-terminal protein but also from poorly conserved homology, even in pairwise alignments of this region or in three-way alignments without the HSV-1 protein (not shown). This C-terminal portion of the HSV-1 protein has been shown not to be required for stimulation of Pol activity, dimerization with Pol, binding to DNA, localization to the nucleus, or replication of the virus in vitro (14, 25, 65), and it is easily separated from the rest of the protein by proteolytic degradation (31). It is especially interesting that although an HSV-1 UL42 protein containing only the N-terminal 315 residues retains Pol-stimulating activity, deletion of the next residue, which forms the end of the N-terminal conserved motif of domain III that we define in Fig. 4B, abolishes activity (65). Thus, it is clear that the C-terminal portions of the PAP homologs form a distinct domain whose function has not been determined but is not likely to be related to Pol accessory activity.

Effect of salt on the activity of the Pol-PAP complex. We observed a stimulation of the HSV-1 and PRV Pol activities with their accessory proteins in the presence of high salt as well as an inhibition of activities by the accessory proteins under low-salt conditions (Fig. 3). Although the assays reported herein were performed with in vitro translation products, we have found similar results with proteins purified from recombinant baculoviruses which express them alone or as a complex (21). The stimulation of Pol activity in high salt may be explained by the lower affinity of the Pol catalytic subunit for DNA compared with that of the Pol-PAP complex (24, 28, 32), coupled with a further destabilization in the presence of high ionic strength. However, it is much more difficult to reconcile an inhibition of activity in the presence of low salt. Our results are similar to those published by Hart and Boehme (32), who also observed an inhibition of the purified HSV-1 Pol activity by the purified accessory protein. However, our observation that the heterologous accessory proteins fail to inhibit the noncognate Pols under these conditions suggests that Pol and PAP physically interact even at low ionic strength and it is the interaction per se which results in inhibition. The inhibition of Pol activity could be explained by the failure of a Pol-PAP complex to bind to DNA at low salt. The low salt would strengthen hydrophobic interactions, perhaps altering the conformation of the proteins, DNA, or both, which could be detrimental to the productive association of the complex with DNA. Alternatively, the complex might bind to DNA so tightly at low salt that the movement required for fork progression would be inhibited. Indeed, a local ionic gradient along the DNA may need to be established to enable the complex or "clamp" to slide along the DNA molecule. The ions present in the absence of exogenously added KCl may be insufficient to establish such a gradient.

Specificity of Pol and PAP interactions. Our results have demonstrated that the PRV Pol and PAP provide functions similar to those of the HSV-1 counterparts. However, despite these functional similarities, the PRV PAP and HSV-1 UL42 cannot substitute for each other. Although the accessory proteins are less conserved than the Pol proteins, the reasonable conservation of the PAPs in the amino two-thirds portions suggests that these proteins are likely to be structurally similar. Structural analysis of other PAPs, most notably the β subunit of *E. coli* Pol III (42) and PCNA, the accessory protein for mammalian cell Pol δ (7, 54), reveals that each forms a sliding clamp over the DNA, the former as a dimer and the latter as a trimer. Despite the structural and functional similarities of these proteins, they share no obvious sequence homology (54). Nevertheless, it is clear from our studies that apparently minor differences in overall structure can affect the ability of accessory proteins to interact with Pol. The specificity by which the accessory proteins interact with their respective Pols and the necessity for this functional interaction in vivo (13, 14, 59, 63) indicate the possibility for the development of antiviral compounds which target this interaction and which are highly effective and specific for blocking viral DNA replication. The assays described herein offer a rapid and facile means for screening the specificity of such compounds.

ACKNOWLEDGMENTS

The work in France was supported by Association pour la Recherche sur le Cancer and by Institut National de la Santé et de la Recherche Médicale. The work in the United States was supported in part by grant GM 34930 from the National Institutes of Health (D.S.P.). H.B. is a doctoral student receiving support from Ministère de la Recherche et de l'Espace (France) and from Ligue Nationale contre le Cancer. S.J.M. was supported in part by a postdoctoral fellowship (F32 AI 08566-02) from the National Institutes of Health.

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