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Cloning, Expression, and Functional Characterization of the Equine Herpesvirus 1 DNA Polymerase and Its Accessory Subunit

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We report the expression and characterization of the putative catalytic subunit (pORF30) and accessory protein (pORF18) of equine herpesvirus 1 DNA polymerase, which are encoded by open reading frames 30 and 18 and are homologous to herpes simplex virus type 1 UL30 and UL42, respectively. In vitro transcription-translation of open reading frames 30 and 18 generated proteins of 136 and 45 kDa, respectively. In vitro-expressed pORF30 possessed basal DNA polymerase activity that was stimulated by pORF18, as measured by DNA polymerase assays in vitro. Purified baculovirus-expressed pORF30 exhibited DNA polymerase activity similar to that of the in vitro-expressed protein, and baculovirus-expressed pORF18 could stimulate both nucleotide incorporation and long-chain DNA synthesis by pORF30 in a dose- and time-dependent manner. The salt optima for activity of both pORF30 and the holoenzyme were substantially different from those for other herpesvirus DNA polymerases. As demonstrated by yeast two-hybrid assays, pORF30 and pORF18 could physically interact, most likely with a 1:1 stoichiometry. Finally, by mutational analysis of the 1,220-residue pORF30, we demonstrated that the extreme C terminus of pORF30 is important for physical and functional interaction with the accessory protein, as reported for UL30 and other herpesvirus DNA polymerases. In addition, a C-proximal region of pORF30, corresponding to residues 1114 to 1172, is involved in binding to, and stimulation by, pORF18. Taken together, the results indicate that pORF30 and pORF18 are the equine herpesvirus 1 counterparts of herpes simplex virus type 1 UL30 and UL42 and share many, but not all, of their characteristics.

Equine herpesvirus 1 (EHV-1) is a pathogen of major importance in horses that can induce a wide spectrum of diseases (1, 45). Like other herpesviruses, EHV-1 establishes a lifelong infection, via a quiescent state known as latency. The outcomes of infection, either following primary infection or reactivation from latency, vary from mild/unapparent respiratory disease to the induction of abortion in pregnant mares and, in extreme cases, neurological disease resulting in paralysis and ultimately death.

EHV-1 is classified on biological grounds as a member of the *Alphaherpesvirinae*, a subfamily of the herpesviruses which is typified by herpes simplex virus type 1 (HSV-1) and also includes varicella-zoster virus (VZV) and pseudorabies virus (PRV) (49). The complete DNA sequence of a pathogenic British isolate (strain Ab4) of EHV-1 was recently determined (56). The genome is approximately 150 kbp in size, is similar in arrangement to the HSV-1, VZV, and PRV genomes, and contains 80 open reading frames (ORFs), corresponding to 76 distinct genes, likely to encode proteins. Comparison of predicted amino acid sequences to those of HSV-1, VZV, and PRV homologs allowed the functions of many EHV-1 proteins to be putatively assigned. However, only a few of these proteins have been studied so far, although the number of characterized proteins is rapidly increasing.

To date, little is known about the mechanism of, and the proteins involved in, EHV-1 replication. The most extensively studied herpesvirus in this area is HSV-1. HSV-1 encodes seven proteins that are required for viral DNA replication and for replication of origin-containing plasmid DNA (38, 63). These proteins include a two-subunit DNA polymerase, which is composed of a catalytic subunit, Pol, or UL30 (47), and an accessory protein, UL42, which stimulates the DNA synthesis activity of UL30 and increases the processivity of the holoenzyme (20, 23). Mutations that specifically disrupt UL30/UL42 interaction ablate virus replication, thus supporting an essential role for this intermolecular association (14, 15, 54, 57). The region in UL30 responsible for binding to UL42 has been localized to the extreme C terminus (14, 17, 39, 54, 57).

In their genomic sequence analysis of the EHV-1 strain Ab4, Telford et al. identified two ORFs, *ORF30* and *ORF18*, that have significant homology to HSV-1 *UL30* and *UL42*, respectively (56). However, nothing is known about the characteristics of the EHV-1 homolog ORFs and the activities of their putative protein products. In order to properly manage the potentially devastating disease that can result from virulent EHV-1 infection, it is important to understand the molecular basis of EHV-1 replication and pathogenesis. Thus, studies on the proteins involved in EHV-1 replication could assist in the development of new anti-EHV-1 compounds. In addition, an

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understanding of the mechanisms of DNA replication in EHV-1 may help elucidate the mechanisms of replication of the closely related human herpesviruses.

Here, we report the cloning and expression of the EHV-1 *ORF30*- and *ORF18*-encoded proteins (which are referred to as pORF30, or Pol, and pORF18, or Pol accessory protein, respectively) both in cell-free transcription-translation reactions and in insect cells infected with recombinant baculoviruses. Also, to investigate whether a direct physical interaction occurs between the proteins encoded by EHV-1 ORFs 30 and 18, we used the yeast two-hybrid system, a genetic method to detect and characterize protein-protein interactions (19). Moreover, the DNA polymerase activity both of in vitro-expressed and of purified baculovirus-expressed pORF30 was tested in the absence and in the presence of pORF18 by DNA polymerase assays in vitro employing different DNA templates and salt concentrations. Finally, to localize the region(s) of interaction between pORF30 and pORF18, we created several pORF30 mutants and tested their abilities to interact with pORF18 in two-hybrid system assays and to be stimulated by pORF18 in DNA polymerase assays. Taken together, the results indicate that pORF30 and pORF18 are the EHV-1 counterparts of the HSV-1 UL30 and UL42 proteins and that they are similar, but not identical, in many aspects.

MATERIALS AND METHODS

Plasmids. The pBS-ORF30 plasmid was constructed by amplifying a 2,360-bp fragment from cosmid 83 (kindly supplied by A. Davison, MRC Virology Unit, Glasgow, United Kingdom), which encodes nucleotides 41,184 to 81,402 of the EHV-1 strain Ab4 genome (56), by PCR with primers 5′-GCATAACTGCAG AAATGGCGGCGCGCGAAC-3′ and 5′-CCCCCCCTCGAGGGATATACCA ACGGTTAG-3′ and cloning of the PCR fragment in the PstI/XhoI sites of pBluescript II KS+ (Stratagene). The pBS-ORF18 plasmid was created by amplifying a 1,280-bp fragment from cosmid 8 (kindly supplied by A. Davison), which encodes nucleotides 1 to 42,740 of the EHV-1 strain Ab4 genome (56), by PCR with primers 5′-TGCAGTCTGCAGTAATGGCTCTTCCTCGCGCG-3′ and 5′-AAAATCCTCGAGACCTAAAAATGCATGGGCTG-3′ and cloning of the PCR fragment in the PstI/XhoI sites of pBluescript II KS+. The pcDNA-ORF30 plasmid was generated by subcloning the BamHI/XhoI fragment of pBS-ORF30 in the BamHI/XhoI sites of pcDNA3.1 (Invitrogen); the pcDNA-ORF18 plasmid was created by subcloning the SmaI/XhoI fragment of pBS-ORF18 in the EcoRV/XhoI sites of pcDNA3.1. The pTM1-ORF30 plasmid was constructed by amplifying the *ORF30* sequence from cosmid 83 with two primers, 5′-CCGCCGCCATGGCGGCGCGCGAACAGG-3′, creating an NcoI site overlapping the 5′ end of the *ORF30* coding sequence, and 5′-CCCCCCCT CGAGGGATATACCAACGGTTAG-3′, creating an XhoI site flanking the 3′ end of *ORF30*. The fragment was then inserted in the NcoI/XhoI sites of plasmid pTM1 (kindly provided by B. Moss, National Institutes of Health, Bethesda, Md.) (44). To create pTM1-ORF18, the *ORF18* coding sequence was amplified from cosmid 8 by PCR with two primers, 5′-CATGTACCATGGCTCTTCCTC GCGCG-3′, creating an NcoI site overlapping the 5′ end of the *ORF18* coding sequence, and 5′-AACGTCCTCGAGCTAAAAATGCATGGGCTG-3′, creating an XhoI site flanking the 3′ end of *ORF18*, and inserted in the NcoI/XhoI sites of pTM1.

To generate the pBTM-ORF30 plasmid, which encodes pORF30 protein fused in frame to the C terminus of *Escherichia coli* LexA (LexA-pORF30), the full-length *ORF30* coding sequence was amplified from cosmid 83 by PCR, using primers 5′-AACGTCGGATCCAAGTGGCGGCGCGCGAACAG-3′ (forward) and 5′-AACGTCCTCGAGATGCATTCAGCTTTGATGGGGAGC-3′ (reverse), and cloned into the BamHI/SalI sites of pBTM116 (43) downstream of the *lexA* gene. The same PCR fragment was also cloned into the BamHI/XhoI sites of pACTII (a gift of D. Shore, Columbia University, New York, N.Y.) to create the pACT-ORF30 plasmid, where pORF30 is fused to the C terminus of the *Saccharomyces cerevisiae* GAL4 activating domain (GAD) (amino acids [aa] 768 to 881) (GAD-pORF30 hybrid). The pBTM-ORF18 plasmid, where pORF18 is fused to the C terminus of LexA (LexA-pORF18 hybrid), was created by amplifying the *ORF18* coding sequence from cosmid 8 by PCR with primers 5′-CATGTAGAATTCGTGGCTCTTCCTCGCGCG-3′ (forward) and 5′-AAC GTCCTCGAGCTGCAGCTAAAAATGCATGGGCTG-3′ (reverse) and cloning the PCR fragment into the EcoRI/SalI sites of pBTM116. To construct the GAD-pORF18 fusion protein, where pORF18 is fused to the C terminus of GAD, the *ORF18* coding sequence was amplified from cosmid 8 by PCR with primers 5′-TAGGTAGAATTCAAGTGGCTCTTCCTCGCGCG-3′ (forward) and 5′-AACGTCCTCGAGCTGCAGCTAAAAATGCATGGGCTG-3′ (reverse) and cloned into the EcoRI/XhoI sites of pACTII, yielding the pACT-ORF18 plasmid.

The plasmids pBTM-ORF30$_{(\Delta 1–400)}$, pBTM-ORF30$_{(\Delta 401–1220)}$, pBTM-ORF30$_{(\Delta 1–1113)}$, pBTM-ORF30$_{(\Delta 1114–1220)}$, pBTM-ORF30$_{(\Delta 1–1187)}$, pBTM-ORF30$_{(\Delta 1188–1220)}$, pBTM-ORF30$_{(\Delta 1218–1220)}$, and pBTM-ORF30$_{(\Delta 1–1113,\ 1173–1220)}$ and the plasmids pACT-ORF30$_{(\Delta 1–400)}$, pACT-ORF30$_{(\Delta 401–1220)}$, pACT-ORF30$_{(\Delta 1–1113)}$, pACT-ORF30$_{(\Delta 1114–1220)}$, pACT-ORF30$_{(\Delta 1–1187)}$, pACT-ORF30$_{(\Delta 1188–1220)}$, pACT-ORF30$_{(\Delta 1218–1220)}$, and pACT-ORF30$_{(\Delta 1–1113,\ 1173–1220)}$, which express deletion mutant pORF30 proteins (residues that have been deleted are indicated within parentheses) fused to LexA or GAD, respectively, were constructed in a manner analogous to that described above for pBTM-ORF30 and pACT-ORF30. A list of the primers used to create these constructs is available at http://www.imbm.unipd.it/Micro1.html or in printed form from the authors on request.

The plasmids pBTM-ORF30$_{(\Delta 1114–1172)}$ and pACT-ORF30$_{(\Delta 1114–1172)}$, wherein nucleotides encoding residues 1114 to 1172 of pORF30 have been deleted, were constructed in two steps. In the first step, a fragment encoding residues 1 to 1113 of pORF30 was amplified from cosmid 83 by PCR using primers 5′-AACGTCGGATCCAAGTGGCGGCGCGCGAACAG-3′ (forward) and 5′-GGGGGTCTTGTGGGGGGTGCGTTTAGGTTGGGGGCG-3′ (reverse), and another fragment, encoding residues 1173 to 1220 of pORF30, was amplified from cosmid 83 by PCR using primers 5′-CGCCCCCAACCTAAAC GCACCCCCCACAAGACCCCC-3′ (forward) and 5′-AACGTCCTCGAGAT GCATTCAGCTTTGATGGGGAGC-3′ (reverse). In the second step, the two resultant PCR products were mixed and reamplified by PCR using primers 5′-AACGTCGGATCCAAGTGGCGGCGCGCGAACAG-3′ (forward) and 5′-AACGTCCTCGAGATGCATTCAGCTTTGATGGGGAGC-3′ (reverse). The final PCR product was then cloned into the BamHI/SalI sites of pBTM116 and into the BamHI/XhoI sites of pACTII.

A similar strategy was used to create plasmids pBTM-ORF30$_{(\Delta 401–1113)}$ and pACT-ORF30$_{(\Delta 401–1113)}$, wherein nucleotides encoding residues 401 to 1113 of pORF30 have been deleted. First, a fragment encoding residues 1 to 400 of pORF30 was amplified from cosmid 83 by PCR using primers 5′-AACGTCGG ATCCAAGTGGCGGCGCGCGAACAG-3′ (forward) and 5′-GGAAACCAG CAGTTTGTTCTGAGTAGCAAG-3′ (reverse), and another fragment, encoding residues 1114 to 1220 of pORF30, was amplified from cosmid 83 by PCR using primers 5′-CTTGCTACTCAGAACAAACTGCTGGTTTCC-3′ (forward) and 5′-AACGTCCTCGAGATGCATTCAGCTTTGATGGGGAGC-3′ (reverse). Then, the two resultant PCR products were mixed and reamplified by PCR using primers 5′-AACGTCGGATCCAAGTGGCGGCGCGCGAACA G-3′ (forward) and 5′-AACGTCCTCGAGATGCATTCAGCTTTGATGGGG AGC-3′ (reverse). The final PCR product was successively cloned into the BamHI/SalI sites of pBTM116 and into the BamHI/XhoI sites of pACTII.

The pTM1-ORF30$_{(\Delta 1114–1220)}$, pTM1-ORF30$_{(\Delta 1114–1172)}$, and pTM1-ORF30$_{(\Delta 1188–1220)}$ plasmids were created by amplifying by PCR the mutant *ORF30* sequences from pBTM-ORF30$_{(\Delta 1114–1220)}$, pBTM-ORF30$_{(\Delta 1114–1172)}$, or pBTM-ORF30$_{(\Delta 1188–1220)}$, respectively, with the same forward primer (5′-CCG CCGCCATGGCGGCGCGCGAACAGG-3′) but with a different reverse primer for each construct [5′-TACTTACTCGAGATGCATTCAGCGTTTAG GTTGGGG-3′ for ORF30$_{(\Delta 1114–1220)}$, 5′-CCCCCCCTCGAGGGATATACCA ACGGTTAG-3′ for ORF30$_{(\Delta 1114–1172)}$, and 5′-AACGTCCTCGAGATGCATT CAGCGCTCAAGCAGTGC-3′ for ORF30$_{(\Delta 1188–1220)}$]. The fragments were then inserted in the NcoI/XhoI sites of vector pTM1.

To create pBTM-UL30, expressing a LexA-UL30 hybrid, the HSV-1 *UL30* gene was amplified from plasmid pE30 (kindly provided by N. D. Stow, MRC Virology Unit, Glasgow, United Kingdom) (55) with primers 5′-GATGTTCCC GGGAATGTTTTCCGGTGGCGGC-3′ (forward) and 5′-GGACGGGTCGAC TCATGCTAGAGTATC-3′ (reverse) and cloned into the SmaI/SalI sites of pBTM116. To create the pACT-UL42 plasmid, expressing a GAD-UL42 hybrid, the HSV-1 *UL42* gene was amplified from plasmid pE42 (kindly provided by N. D. Stow) (55) with primers 5′-GATGTTGGATCCAAATGACGGATTCCC CTGGC-3′ (forward) and 5′-GGACGGCTCGAGTCAGGGGAATCCAAAA CC-3′ (reverse) and cloned into the BamHI/XhoI sites of pACTII.

All constructs were sequenced with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) to confirm the absence of undesired mutations.

In vitro transcription-translation. The TNT-coupled reticulocyte lysate system with T7 RNA polymerase (Promega) was used to transcribe and translate the *ORF30* and *ORF18* coding sequences from either pBS-ORF30 and pBS-ORF18, pcDNA-ORF30 and pcDNA-ORF18, or pTM1-ORF30 and pTM1-ORF18, respectively, in the presence of [^{35}S]methionine (Amersham Pharmacia Biotech), according to the manufacturer's instructions. The reaction products were analyzed by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–7.5% PAGE) and autoradiography. The molecular mass of the translated products was determined by comparing the migration rate of the ^{35}S-labeled proteins with the migration rate of a set of molecular mass markers (SDS-PAGE standards, high range; Bio-Rad) that were stained with Coomassie blue after they were run in parallel with the ^{35}S-labeled material.

Yeast manipulation and β-Gal assays. Growth media and standard methods for manipulating yeast cells were as described by Rose et al. (50). The yeast strain *S. cerevisiae* CTY10-5d (*MAT***a** *ade2-1 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3*::*lexA op-lacZ*) (43) was transformed with plasmid DNA by the lithium acetate method of Schiestl and Giest (51). Transformed cells were assayed for expression of the *lacZ* reporter gene by β-galactosidase (β-Gal) filter assays as described previously (7). In these assays, β-Gal expression was scored as follows: +, strong blue color detected after 3 to 5 h of incubation; ±, light blue color detected after 8 to 12 h of incubation; −, no signal detected after 24 h of incubation.

To quantitate β-Gal expression, the method of Breeden and Nasmyth (6), normalizing activities to total protein content, was used. One β-Gal unit corresponds to $10^3 \times A_{420}/(t \times V \times p)$, where A_{420} is the absorbance at 420 nm, t is the reaction time (min), V protein is the protein extract volume (ml), and p is the total protein concentration (mg/ml) (6). The reported values represent the mean β-Gal activity of 3 to 4 colonies from at least three independent transformations ± standard deviation.

Cells and viruses. *Spodoptera frugiperda* (Sf9) cells were maintained in TC100 medium (Invitrogen Life Technologies) supplemented with 5% (vol/vol) fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and neomycin (50 μg/ml).

The recombinant *Autographa californica* baculovirus expressing pORF30 under the control of the polyhedrin promoter was generated as follows. The *ORF30* coding sequence was amplified by PCR from cosmid 83. The primers used were: 5′-ATCGTCGGATCCATGGCGGCGCGCGAAC-3′ and 5′-ACCGTCCTGC AGTCAGCTTTGATGGGG-3′, which contained BamHI and PstI sites, respectively. The PCR-generated fragment was then cloned into the BamHI/PstI sites of the baculovirus transfer vector pAcCL29.1 (32) downstream of the polyhedrin promoter to generate plasmid pAcCL-ORF30. The entire fragment was sequenced to confirm the presence of the authentic *ORF30* coding sequence. The transfer plasmid pAcCL-ORF30 was cotransfected with Bsu36I-cleaved DNA (Clontech) of the parental baculovirus AcPAK6 (4) into Sf9 cells, and the resultant recombinant baculovirus AcORF30 was isolated as described by Kitts et al. (29).

The recombinant baculovirus expressing pORF18 was generated with a similar strategy. The *ORF18* coding sequence was amplified by PCR from cosmid 8 using the primers 5′-ATCGTCTCTAGAATGGCTCTTCCTCGC-3′ and 5′-CACGT CCTGCAGCTAAAAATGCATGGG-3′, which contained XbaI and PstI sites, respectively. The PCR-generated fragment was then cloned into the XbaI/PstI sites of pAcCL29.1 downstream of the polyhedrin promoter to generate plasmid pAcCL-ORF18. The transfer plasmid pAcCL-ORF18 was cotransfected with Bsu36I-cleaved DNA (Clontech) of the parental baculovirus AcPAK6 into Sf9 cells to create the recombinant baculovirus AcORF18.

The presence of the *ORF30* or *ORF18* gene in the recombinant baculovirus genome was confirmed by PCR analysis of DNA extracted from Sf9 cells infected with AcORF30 or AcORF18, respectively. Preparation and titration of virus stocks were described previously (40).

The recombinant baculoviruses AcUL30 and AcUL42, which express HSV-1 UL30 and UL42, respectively, have been described (53, 54).

Protein purification. HSV-1 UL30 was purified from Sf9 cells infected with AcUL30 first by phosphocellulose and then by DNA cellulose chromatography, as described previously (23, 39). The HSV-1 UL42 protein was purified from Sf9 cells infected with AcUL42 as reported previously (23, 39).

Recombinant pORF30 was purified from Sf9 cells infected with AcORF30 as described for HSV-1 UL30. Recombinant pORF18 was purified from Sf9 cells infected with AcORF18 by a procedure similar to that used for HSV-1 UL42, with the following modifications. Sf9 cells (2.5×10^9) were infected with AcORF18 at a multiplicity of infection of 10 PFU per cell and harvested at 24 h postinfection. Infected cells were washed three times with cold phosphate-buffered saline, resuspended in a mixture of 20 mM HEPES (pH 7.9), 0.5 mM dithiothreitol (DTT), 0.5 mM $MgCl_2$, and 10 mM $NaHSO_3$, containing protease inhibitors (complete protease inhibitor cocktail tablets; Roche Applied Science), and homogenized. The extract was then centrifuged for 5 min at 4,000 rpm at 4°C, and the pellet was resuspended in the same buffer, but containing 3.4 M NaCl, and incubated on ice for 10 min. Insoluble material was removed by centrifugation for 1 h at 40,000 rpm at 4°C. The cell extract was successively dialyzed extensively against a mixture of 20 mM HEPES (pH 7.6), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 10% glycerol, containing protease inhibitors (complete protease inhibitor cocktail tablets; Roche Applied Science). Protein purification was conducted at room temperature with the assistance of an AKTApurifier fast-protein liquid chromatography system (Amersham Pharmacia Biotech), and all fractions were collected at 0°C. For the first purification step, the protein extract was applied to a 10-ml double-stranded DNA cellulose column (Sigma) equilibrated with 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, and 10% glycerol. Proteins were eluted with a linear salt gradient from 50 mM to 0.7 M NaCl. The presence of pORF18 protein in column fractions was determined by SDS–10% PAGE analysis. After dialysis against 20 mM Tris-HCl (pH 8.0) containing 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, and 10% glycerol, plus protease inhibitors (complete protease inhibitor cocktail tablets; Roche Applied Science), the sample was loaded onto a Mono-Q column (HR 5/5; Amersham Pharmacia Biotech) equilibrated with 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, and 10% glycerol and eluted with a gradient of 50 mM to 0.5 M NaCl. Fractions were analyzed by SDS–10% PAGE, and those containing purified pORF18 were pooled together, dialyzed against 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, and 10% glycerol, and stored at −80°C until use.

The purity of the final pORF30 and pORF18 preparations was evaluated by analyzing 1 to 2 μg of purified protein by 5% and 10% SDS-PAGE, respectively, followed by Coomassie blue staining.

DNA polymerase assays. Basal DNA polymerase activity of pORF30 and stimulation of pORF30 activity by pORF18 was assayed by measuring the incorporation of [^{3}H]dTTP into either a poly(dA)-oligo(dT) or calf thymus DNA template, as previously described (37), using 5 μl of in vitro transcribed-translated pORF30 in the absence or in the presence of 5 μl of in vitro transcribed-translated pORF18 or 200 fmol of purified baculovirus-expressed pORF30 in the absence or in the presence of different amounts of purified baculovirus-expressed pORF18. In some experiments, 200 fmol of purified baculovirus-expressed HSV-1 UL30 and/or UL42 were used. All assays were performed in the presence of 150 mM KCl, except in experiments testing the effect of salt concentration on the activity of pORF30 and of the pORF30-pORF18 complex, wherein the KCl concentration was varied as indicated. The concentration of NaCl carried over into the assays from the pORF30 or pORF30-pORF18 protein complex solutions was ≤1 mM. The reported values represent the average of data from at least three independent experiments, each performed in triplicate. Long-chain DNA synthesis by pORF30 in the presence of pORF18 was assayed as reported previously (33) with 200 fmol of purified baculovirus-expressed pORF30 and 100 or 200 fmol of purified baculovirus-expressed pORF18 in a final volume of 25 μl. Reactions were carried out at 37°C for 60 min. The molecular size of the DNA products was determined by comparing the migration rate of the ^{32}P-labeled products with the migration rate of a set of molecular mass markers (DNA Molecular Weight Marker XIII; Roche Applied Science) that were stained with ethidium bromide after they were run in parallel with the ^{32}P-labeled material.

Alignment of the pORF30 sequence with alphaherpesvirus protein homologs. The protein sequences of pORF30 and alphaherpesvirus homologs were obtained by translation of sequences available in the GenBank-EMBL database (EHV-1 pORF30 [56], HSV-1 UL30 [41], VZV gene 28 protein [13], PRV Pol [3, 30], and bovine herpesvirus 1 [BHV-1] Pol [42]). Protein sequences were aligned by use of the CLUSTAL W multiple sequence alignment program (58). Conservation was determined by chemical and structural considerations according to the groupings DE, KRH, AVFPMILW, and STYHCNGQ.

RESULTS

Analysis of EHV-1 ORF30 and ORF18 translation products. In order to characterize the proteins encoded by EHV-1 *ORF30* and *ORF18*, the two coding sequences were cloned and expressed in an in vitro-coupled transcription-translation system. Initially, *ORF30* and *ORF18* were cloned in pBluescript II, under the control of a T7 promoter. However, while a band corresponding to the expected molecular mass of the pORF18 protein (~45 kDa) was obtained from an in vitro expression

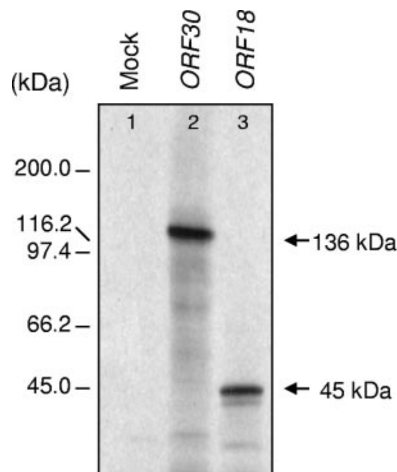

FIG. 1. In vitro transcription-translation products of the EHV-1 *ORF30* and *ORF18* genes. The *ORF30* and *ORF18* coding sequences were introduced into the pTM1 expression vector under the control of a T7 promoter and the EMV 5′ UTR and were in vitro expressed with the Promega T7-TNT coupled transcription-translation system. The [^{35}S]methionine-labeled *ORF30* and *ORF18* translation products were fractionated by SDS–7.5% PAGE and examined by autoradiography. Mock, translation from the pTM1 vector lacking a coding insert. The molecular masses of protein markers, in kilodaltons, analyzed on the same gel are indicated on the left; the apparent masses of *ORF30* and *ORF18* translation products are shown on the right.

reaction with the pBS-ORF18 construct, no significant protein expression from the pBS-ORF30 plasmid was observed (data not shown). Similar results were obtained when *ORF30* and *ORF18* were cloned in pcDNA3.1, which in addition to a T7 promoter possesses signals of bovine growth hormone gene, allowing efficient transcription termination and polyadenylation of mRNA (22). Furthermore, no significant increase in translation efficiency of either *ORF30* or *ORF18* was observed by varying the amount of template plasmid or the temperature and time of the reaction (data not shown).

It has been shown that the 5′ untranslated region (UTR) of eukaryotic genes can significantly affect the efficiency of both in vitro (28) and in vivo (31) mRNA translation. Some viral 5′ UTRs, such as that from encephalomyocarditis virus (EMV) (27) or alfalfa mosaic virus (28), and some nonviral 5′ UTRs, such as that of *Xenopus laevis* β-globin (18), have been shown to enhance in vitro translation. In contrast, it has been reported that in vitro translation of human cytomegalovirus (HCMV) DNA polymerase, UL54, proceeds very inefficiently when driven by its original 5′ UTR (64). The EMV 5′ UTR has been shown to substantially enhance in vitro translation of *UL54* when placed immediately upstream from the ATG codon (10). Thus, to try to increase in vitro expression of pORF30, *ORF30* was cloned in the pTM1 vector, which contains the EMV 5′ UTR downstream from the T7 promoter (44). In parallel, *ORF18* was also cloned in this plasmid. As shown in Fig. 1, a protein of approximately 45 kDa, corresponding to the predicted 405-aa pORF18, was synthesized from pTM1-ORF18 (lane 3). The expression level of pORF18 from pTM1 was comparable to that obtained from pBluescript II and pcDNA3.1 under similar experimental conditions (data not shown). In contrast, pORF30 expression appeared to be dramatically enhanced by the EMV 5′ UTR, as a protein of approximately 136 kDa, corresponding to the predicted 1,220-aa pORF30, was detected by SDS-PAGE analysis of an in vitro expression reaction with pTM1-ORF30 (Fig. 1, lane 2).

Thus, both *ORF30* and *ORF18* can drive the synthesis of protein products, whose molecular masses are in good agreement with those predicted from the sizes of the ORFs.

The pORF30 and pORF18 proteins form a heterodimeric complex. To test whether pORF30 can physically interact with pORF18, the yeast two-hybrid system (THS) was employed. We used a version of the THS wherein pORF30 and pORF18 are fused to LexA (an *E. coli* DNA-binding protein) or to the transcription-activating domain (GAD) of the *S. cerevisiae* GAL4 protein (aa 768 to 881) and are expressed in a yeast strain containing eight copies of the LexA-binding sequence (*lexO*) upstream of the *lacZ* reporter gene. This variant of the yeast THS, among several proposed, is particularly sensitive in detecting protein-protein interactions (52).

The recombinant plasmids pBTM-ORF30 and pBTM-ORF18, encoding the LexA-pORF30 and LexA-pORF18 hybrid proteins, respectively, and plasmids pACT-ORF30 and pACT-ORF18, encoding the GAD-pORF30 and GAD-pORF18 hybrid proteins, respectively, were introduced individually, or in pair-wise combinations, into the yeast cells. The positive control was represented by yeast cells transformed with plasmids encoding LexA and GAD fusions to HSV-1 UL30 and UL42, respectively, which have been previously shown by other techniques to interact (16, 21, 39, 57). We found that yeast colonies expressing the LexA-pORF30 and GAD-pORF18 hybrids or the LexA-pORF18 and GAD-pORF30 hybrids showed β-Gal expression, while yeast cells expressing only one of the fusion proteins exhibited no detectable β-Gal activity in filter assays (Table 1). Quantitative mea-

TABLE 1. Analysis of pORF30-pORF18 interaction by the yeast THS[a]

Hybrid		β-Gal expression (units ± SD)[b]
DNA-binding domain fusion	Activation domain fusion	
LexA-pORF30		− (<1)
	GAD-pORF30	− (<1)
LexA-pORF18		− (<1)
	GAD-pORF18	− (<1)
LexA-pORF30	GAD-pORF18	+ (8,182.4 ± 318.2)
LexA-pORF18	GAD-pORF30	+ (7,435.8 ± 467.1)
LexA-UL30	GAD-UL42	+ (9,764.7 ± 223.8)
LexA-UL30	GAD-pORF18	− (<1)
LexA-pORF30	GAD-UL42	− (<1)
LexA-pORF30	GAD-pORF30	− (<1)
LexA-pORF18	GAD-pORF18	− (<1)

[a] EHV-1 pORF30 and pORF18 or HSV-1 UL30 and UL42 proteins were fused to the C terminus of LexA (DNA-binding domain) or of the GAL4 activation domain (GAD) and assayed for interaction by qualitative and quantitative β-Gal assays as described in Materials and Methods.

[b] +, strong blue color detected in β-Gal filter assays after 3 to 5 h of incubation; −, no signal detected after 24 h of incubation. Values within parentheses are β-Gal units for three or four yeast colonies from at least three independent transformations.

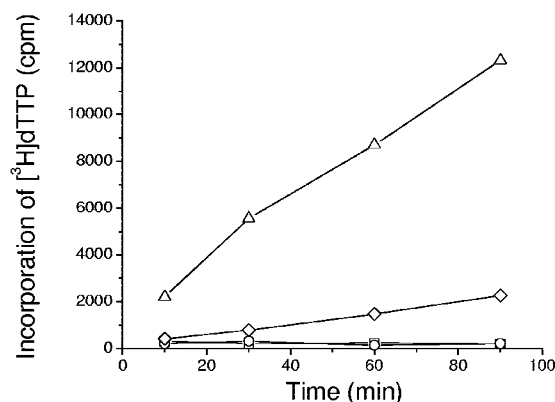

FIG. 2. pORF30 possesses DNA polymerase activity which is stimulated by pORF18. The DNA synthesis activity of in vitro-transcribed-translated pORF30, pORF18, or pORF30 plus pORF18 was analyzed by measuring the incorporation of [^{3}H]dTTP into a poly(dA)-oligo(dT) template. Mock represents the DNA polymerase activity of the translation reaction from the pTM1 vector lacking a coding insert. □, mock; ○, pORF18; ◇, pORF30; △, pORF30 plus pORF18.

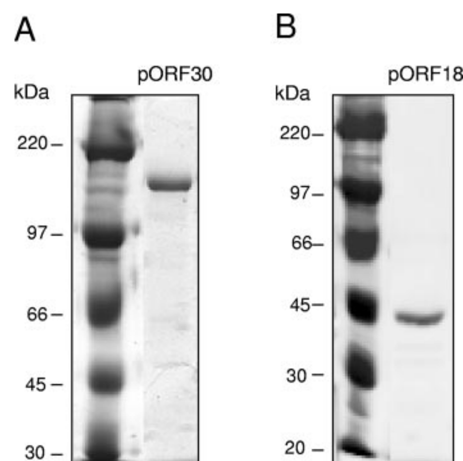

FIG. 3. Expression and purification of recombinant baculovirus-expressed pORF30 and pORF18. Proteins were expressed in insect cells infected with recombinant baculoviruses and purified by column chromatography as described in Materials and Methods. Samples after the final step of pORF30 (A) and pORF18 (B) purification were analyzed by 5% and 10% SDS-PAGE, respectively. The positions of the molecular mass markers are indicated on the left.

surement of β-Gal activity confirmed that the pORF30-pORF18 interaction did occur, compared with the UL30/UL42 positive control (Table 1).

To demonstrate the specificity of the pORF30-pORF18 interaction, pORF18 and pORF30 were tested for the ability to interact with HSV-1 UL30 and UL42, respectively. As shown in Table 1, pORF30 was unable to bind UL42, and pORF18 did not interact with UL30.

To investigate the possibility that pORF18 might dimerize, as recently reported for other herpesvirus DNA polymerase accessory subunits (2, 9), we assayed whether the LexA-ORF18 and GAD-ORF18 fusion proteins could interact in yeast THS assays. As shown in Table 1, pORF18 did not interact with itself, as indicated by the lack of β-Gal activity in cells expressing LexA-pORF18 in combination with GAD-pORF18. Analogously, the LexA-pORF30 and GAD-pORF30 hybrids did not interact (Table 1), whereas HCMV UL44-UL44 self-interaction was detected in similar THS assays (A. Loregian, E. Sinigalia, and G. Palù, unpublished results), in keeping with the observation that UL44 can homodimerize (2).

Taken together, these data indicate that a physical interaction between pORF30 and pORF18 does occur and that pORF30 and pORF18 most likely form a heterodimeric complex with a 1:1 stoichiometry.

The pORF30 protein possesses DNA polymerase activity that is stimulated by pORF18. To determine whether pORF30 and pORF18, which are the putative catalytic subunit and processivity factor of EHV-1 DNA polymerase, respectively, are able to function as predicted, we performed DNA polymerase assays with in vitro transcription-translation products of the genes. The DNA synthesis activity of pORF30 in the absence or in the presence of pORF18 was assayed by an in vitro filter-based DNA polymerase assay which employs poly(dA)-oligo(dT) as the template (37). Figure 2 shows that the in vitro-expressed pORF30 protein possesses basal DNA polymerase activity even in the absence of the accessory protein. In contrast, no significant activity was observed either in mock-translated reactions or in translation products of the *ORF18* gene. However, when we mixed the *ORF30* and *ORF18* translation products, we observed up to sevenfold stimulation of the nucleotide incorporation activity of pORF30 compared with the activity of pORF30 alone. Thus, the EHV-1 gene designated *ORF30* encodes a protein that possesses DNA polymerase activity and the *ORF18*-encoded protein stimulates such an activity.

To further characterize the activity of pORF30 alone and of the pORF30-pORF18 complex, we created recombinant baculoviruses containing *ORF30* and *ORF18* coding sequences and purified the pORF30 and pORF18 proteins from insect cell cultures infected with such viruses. SDS-PAGE analysis of purified baculovirus-expressed pORF30 and pORF18 is shown in Fig. 3. Both pORF30 and pORF18 appeared to be substantially homogeneous, as no other proteins at the final stage of purification were detectable by Coomassie blue staining. The functional properties of recombinant baculovirus-expressed pORF30 and pORF18 were then compared with those of the in vitro-transcribed-translated proteins by measuring the rate of incorporation of labeled dTTP into different templates by pORF30 both in the absence and in the presence of different amounts of pORF18 (Fig. 4). In this assay, baculovirus-expressed pORF30 exhibited DNA polymerase activity as well as the in vitro-expressed protein. Moreover, purified pORF18 stimulated the nucleotide incorporation activity of pORF30 with either poly(dA)-oligo(dT) (Fig. 4) or calf thymus DNA (data not shown) used as a template. This stimulation was dependent on the amount of pORF18 added. The maximal stimulation (approximately 11-fold) was calculated to occur at a 1:1 molar ratio of pORF30 and pORF18.

To examine the specificity of pORF30 and pORF18 functional interaction, the EHV-1 proteins were tested for the ability to function with HSV-1 UL30 and UL42. As expected (23), purified baculovirus-expressed UL30 alone possessed basal DNA polymerase activity, and addition of purified baculovirus-expressed UL42 stimulated such an activity (Fig. 5).

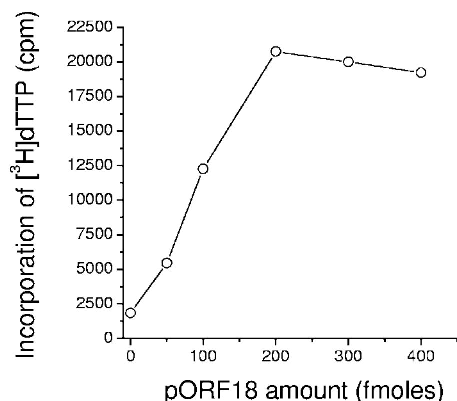

FIG. 4. Stimulation of DNA polymerase activity of purified baculovirus-expressed pORF30 by pORF18. The effects of different amounts of purified pORF18 on the activity of 200 fmol of pORF30 were examined by measuring the incorporation of [^{3}H]dTTP into a poly(dA)-oligo(dT) template in a 60-min reaction.

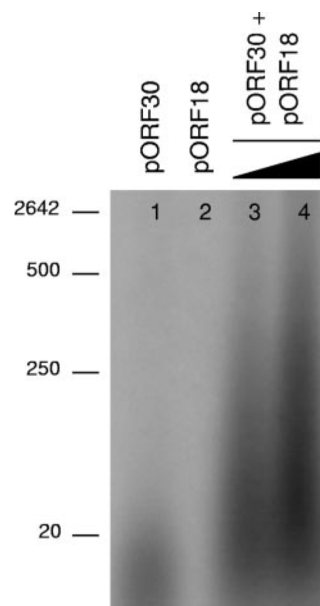

FIG. 6. pORF18 enables pORF30 to synthesize long-chain DNA products. Long-chain DNA synthesis by purified baculovirus-expressed pORF30 in the absence and in the presence of purified baculovirus-expressed pORF18 was assayed by measuring the incorporation of [^{32}P]TTP on a poly(dA)-oligo(dT) template. The reaction products were visualized by autoradiography following electrophoresis on a 4% alkaline agarose gel. Positions of DNA size markers (bases) and the length of short DNA products are indicated at left. Lane 1 contains 200 fmol of pORF30 alone; lane 2 contains 200 fmol of pORF18 alone; and lanes 3 and 4 contain 200 fmol of pORF30 plus 100 or 200 fmol of pORF18, respectively.

However, UL42 was not able to stimulate pORF30 activity, nor was pORF18 able to enhance UL30-mediated nucleotide incorporation activity (Fig. 5). These results suggest that pORF30 and pORF18 are specific for one another, as they cannot be functionally replaced by homologous alphaherpesvirus DNA polymerase subunits.

We then tested the ability of pORF18 to stimulate long-chain DNA synthesis by pORF30 by measuring the incorporation of radionucleotides into the oligo(dT)-poly(dA) primer/template and analyzing the resulting products on an alkaline agarose gel. As shown in Fig. 6, only limited synthesis of short DNA products was detectable after incubation of this template with purified baculovirus-expressed pORF30 in the absence of pORF18 (lane 1), and no extension of the oligo(dT) primer was observed when only pORF18 was included in the reaction (lane 2). In contrast, addition of pORF18 to pORF30 resulted in synthesis of long-chain DNA products (lanes 3 and 4), in a manner that was dependent on the pORF18 amount. With constant amounts of pORF30 and pORF18, the stimulation of pORF30-mediated long-chain DNA synthesis by pORF18 was also time dependent. As reported for long-chain DNA synthesis by the HSV-1 UL30-UL42 complex (17, 24), formation of long DNA products by pORF30-pOR18 could be detected after a 5- to 10-min incubation and reached a maximum within 60 min (not shown). Very similar results were obtained when long-chain DNA synthesis by in vitro-expressed pORF30 and pORF18 was assayed in the same manner (data not shown).

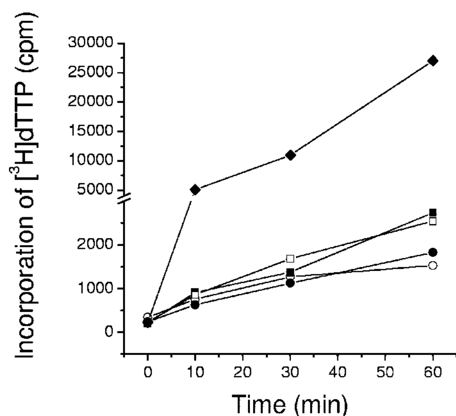

FIG. 5. The functional interaction between the EHV-1 DNA polymerase subunits, pORF30 and pORF18, is specific. Stimulation of DNA polymerase activity of pORF30 by UL42, the HSV-1 DNA polymerase accessory protein, and stimulation of UL30, the HSV-1 DNA polymerase catalytic subunit, by pORF18 were examined by measuring the incorporation of [^{3}H]dTTP into a poly(dA)-oligo(dT) template by individual or combined proteins. As a control, stimulation of UL30 by UL42 was also assayed. Equimolar amounts (200 fmol) of purified baculovirus-expressed proteins were used in these assays. ●, pORF30 alone; ○, pORF30 plus UL42; ■, UL30 alone; □, UL30 plus pORF18; ◆, UL30 plus UL42.

Finally, because the activity of herpesvirus DNA polymerases has been shown to be stimulated in the presence of high salt concentrations (10, 25), we investigated the sensitivity of pORF30 alone and of the pORF30-pORF18 complex to salt concentration. The DNA polymerase activity of purified baculovirus-expressed pORF30 and pORF18 proteins was assayed in the presence of increasing concentrations of KCl. As previously observed for HSV-1 UL30 (25), we found the activity of pORF30 to be extremely sensitive to salt in the absence of its accessory protein, with a sharp salt optimum at 50 mM KCl (Fig. 7). Indeed, with 50 mM KCl, pORF30 exhibited more than 40 times the activity found in the presence of 150 mM KCl (Fig. 7). In the presence of pORF18, the dependence curve followed a much shallower profile, with maximum activity in the absence of exogenously added KCl and decreasing activity

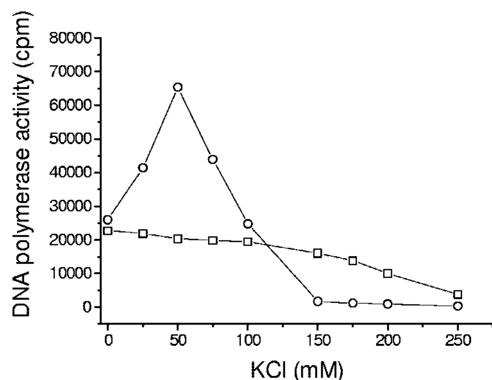

FIG. 7. Effects of salt concentration on DNA polymerase activities of pORF30 and the pORF30-pORF18 complex. The DNA polymerase activities of pORF30 alone (○) and of pORF30 plus pORF18 at an equimolar ratio (□), determined by filter-based DNA polymerase assays employing poly(dA)-oligo(dT) as the template, are plotted against the concentration of KCl present in the reaction mixtures. NaCl (1 mM or less) was carried over into assays from protein solutions.

observed when increasing amounts of KCl were added (Fig. 7). However, the net effect was that the accessory protein inhibited the activity of pORF30 at a low salt concentration and stimulated its activity in the presence of a high salt concentration. The actual stimulatory effect of pORF18 on the activity of the catalytic subunit was detectable at a KCl concentration higher than 120 mM. The maximal effect, approximately 11-fold stimulation, was observed at 150 mM KCl. On the contrary, at a low salt concentration (50 mM KCl), the presence of the accessory protein decreased the DNA polymerase activity of pORF30 by about 70%. Thus, as for other herpesvirus DNA polymerases, both the DNA polymerase activity of the EHV-1 catalytic subunit alone and that of the holoenzyme complex appear to be dependent on the salt concentration; however, differences between the EHV-1 enzyme and other herpesvirus DNA polymerases in salt-dependence profiles do exist (see discussion).

The pORF30 protein physically interacts with pORF18 through both a C-proximal and a C-terminal region. We next wished to identify the region(s) of pORF30 that is important for binding to pORF18. To this aim, several pORF30 deletion mutants were generated, fused to both LexA and GAD, and tested for the ability to interact with pORF18 by the means of THS assays. A schematic diagram of each mutant is shown in Fig. 8. Control experiments to test whether all mutant LexA-pORF30 and GAD-pORF30 fusion proteins were expressed in yeast were performed by Western blot analysis of yeast transformant protein extracts with anti-LexA or anti-GAD antibody. Protein bands of the expected molecular mass were evidenced for all pORF30 mutants (data not shown).

In order to localize the pORF18-binding site within the

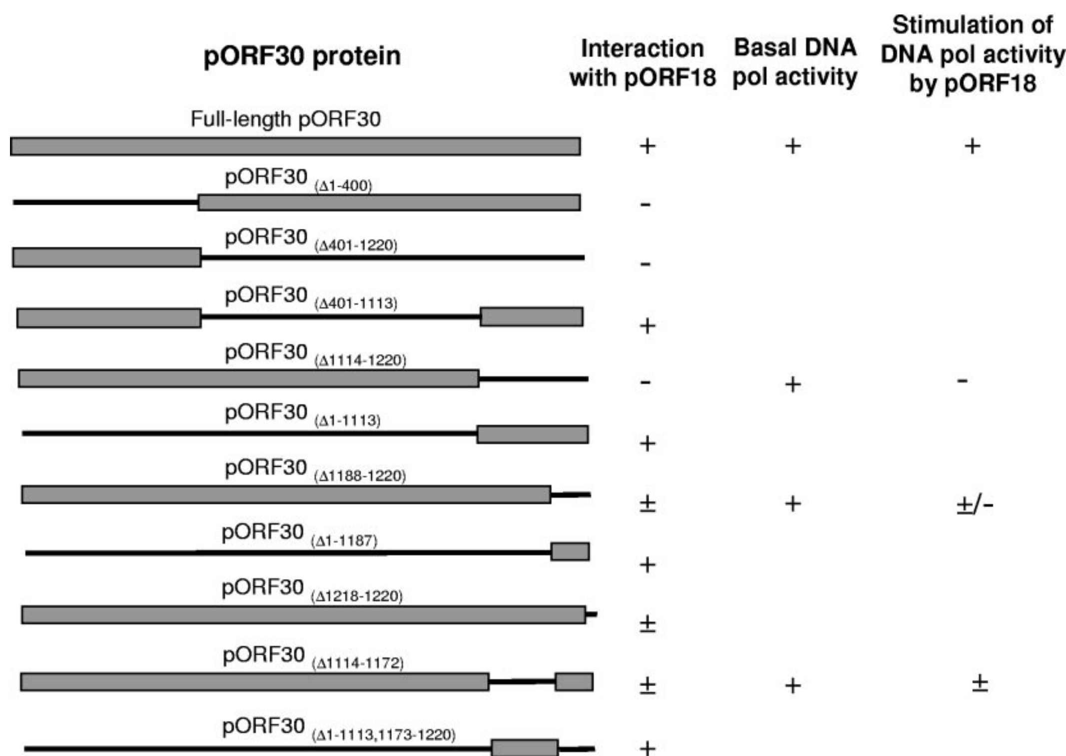

FIG. 8. Schematic representation and summary of the properties of full-length pORF30 and pORF30 deletion mutants. Several pORF30 mutants were created by deleting different portions of the EHV-1 *ORF30* coding sequence (pORF30 residues that have been deleted are indicated within parentheses). Deleted portions of pORF30 are represented by black lines, while regions still expressed are depicted as gray bars. The representations of the mutants are not drawn to scale. The ability of full-length and mutant pORF30 proteins to physically interact with pORF18, as determined by yeast two-hybrid assays (Table 2), and their catalytic activity both in the absence and in the presence of pORF18, as measured by DNA polymerase assays (Fig. 9), are scored as follows: +, wild-type levels of activity; ±, partially impaired activity; ±/−, almost completely impaired activity; −, no detectable activity.

TABLE 2. Analysis of the interaction of pORF30 mutants with pORF18 by the yeast THS[a]

Hybrid		β-Gal expression[b]
DNA-binding domain fusion	Activation domain fusion	
LexA-pORF30$_{(\Delta 1\text{–}400)}$	GAD-pORF18	−
LexA-pORF30$_{(\Delta 401\text{–}1220)}$	GAD-pORF18	−
LexA-pORF30$_{(\Delta 401\text{–}1113)}$	GAD-pORF18	+
LexA-pORF30$_{(\Delta 1114\text{–}1220)}$	GAD-pORF18	−
LexA-pORF30$_{(\Delta 1\text{–}1113)}$	GAD-pORF18	+
LexA-pORF30$_{(\Delta 1188\text{–}1220)}$	GAD-pORF18	±
LexA-pORF30$_{(\Delta 1\text{–}1187)}$	GAD-pORF18	+
LexA-pORF30$_{(\Delta 1218\text{–}1220)}$	GAD-pORF18	±
LexA-pORF30$_{(\Delta 1114\text{–}1172)}$	GAD-pORF18	±
LexA-pORF30$_{(\Delta 1\text{–}1113,\ 1173\text{–}1220)}$	GAD-pORF18	+
LexA-pORF18	GAD-pORF30$_{(\Delta 1\text{–}400)}$	−
LexA-pORF18	GAD-pORF30$_{(\Delta 401\text{–}1220)}$	−
LexA-pORF18	GAD-pORF30$_{(\Delta 401\text{–}1113)}$	+
LexA-pORF18	GAD-pORF30$_{(\Delta 1114\text{–}1220)}$	−
LexA-pORF18	GAD-pORF30$_{(\Delta 1\text{–}1113)}$	+
LexA-pORF18	GAD-pORF30$_{(\Delta 1188\text{–}1220)}$	±
LexA-pORF18	GAD-pORF30$_{(\Delta 1\text{–}1187)}$	+
LexA-pORF18	GAD-pORF30$_{(\Delta 1218\text{–}1220)}$	±
LexA-pORF18	GAD-pORF30$_{(\Delta 1114\text{–}1172)}$	±
LexA-pORF18	GAD-pORF30$_{(\Delta 1\text{–}1113,\ 1173\text{–}1220)}$	+

[a] Mutant pORF30 proteins or pORF18 were fused to the C terminus of LexA (DNA-binding domain) or of the GAL4 activation domain (GAD) and assayed for interaction by β-Gal filter assays as described in Materials and Methods.

[b] +, strong blue color detected after 3 to 5 h of incubation; ±, light blue color detected after 8 to 12 h of incubation; −, no signal detected after 24 h of incubation.

1,220-residue pORF30 protein, we initially deleted ample portions of *ORF30*. Deletion of the N-terminal one-third of pORF30 (aa 1 to 400) completely impaired the ability of pORF30 to interact with pORF18, suggesting that this region may contain sequences important for pORF18 binding (Table 2). However, the fragment corresponding to residues 1 to 400 of pORF30 [pORF30$_{(\Delta 401\text{–}1220)}$] did not interact with pORF18 in yeast THS assays (Table 2), suggesting that our inability to observe physical interactions between the pORF30$_{(\Delta 1\text{–}400)}$ deletion mutant and pORF18 was likely due to improper folding of the mutant protein in yeast cells. We then deleted a large, central portion of *ORF30* encoding residues 401 to 1113 of pORF30 in such a fashion that the reading frame was maintained between the amino- and carboxyl-terminal fragments. The pORF30$_{(\Delta 401\text{–}1113)}$ mutant still interacted with pORF18 (Table 2), indicating that the central region is probably not involved in pORF18 binding. Moreover, these data suggested that the pORF18-binding site could reside in the remaining, C-terminal part of pORF30. To test this hypothesis, a mutant lacking the 107 C-terminal residues of pORF30 [pORF30$_{(\Delta 1114\text{–}1220)}$] was constructed and assayed for ability to bind pORF18. As expected, this pORF30 mutant did not interact with pORF18, suggesting that the fragment of pORF30 corresponding to residues 1114 to 1220 could indeed be involved in binding to pORF18. In keeping with this idea, a mutant that only expresses the C-terminal 107 residues of pORF30 [pORF30$_{(\Delta 1\text{–}1113)}$] was able to interact with pORF18 in yeast THS assays (Table 2). Taken together, these results indicate that residues 1114 to 1220 of pORF30 contain a region(s) that is important for interaction with pORF18.

Therefore, we then tried to map the sequence(s) within the 107 C-terminal residues of pORF30 that is necessary and sufficient for interacting with pORF18. Several studies have demonstrated that the catalytic subunit of herpesvirus DNA polymerases interacts with the accessory protein through the extreme C terminus (3, 17, 37, 39), although possible contributions by more C-proximal regions have not been completely excluded. Comparison of the pORF30 amino acid sequence with that of HSV-1 UL30 showed that the last 33 residues of pORF30 present some homology (36% identity, 54% similarity) with the 27 C-terminal amino acids of UL30, which have been previously shown to be important for physical and functional interactions between UL30 and UL42 (39). Thus, to investigate whether the 33 C-terminal residues of pORF30 might play a role in pORF18 binding, we created a pORF30 deletion mutant lacking this region [pORF30$_{(\Delta 1188\text{–}1220)}$] and tested its ability to interact with pORF18 by yeast THS assays. We found that the pORF30$_{(\Delta 1188\text{–}1220)}$ mutant still binds pORF18 but more weakly than the wild-type protein, as indicated by the lower β-Gal expression (Table 2). Quantitative measurement of β-Gal activity confirmed such an observation (data not shown). These data suggest that the extreme C terminus of pORF30 may be involved in pORF18 binding. Consistent with this interpretation, a fragment corresponding to the 33 C-terminal residues of pORF30 [pORF30$_{(\Delta 1\text{–}1187)}$] was able to interact with pORF18 in yeast two-hybrid assays.

The results obtained with the pORF30$_{(\Delta 1188\text{–}1220)}$ mutant also suggested that other regions within the 107 C-terminal residues of pORF30 might have a role in binding to pORF18. By comparing the amino acid sequences of the DNA polymerase catalytic subunit of a number of human and animal herpesviruses, Berthomme et al. recently identified a C-proximal domain that is well conserved within the alphaherpesvirus group (3). This domain, which in pORF30 extends from residue 1114 to 1172, is included in the HSV-1 UL30 region initially described by Digard and Coen (16) to be necessary and sufficient for stable association with UL42. We hence tested the effect of the deletion of this domain [pORF30$_{(\Delta 1114\text{–}1172)}$] on pORF18 binding. As shown in Table 2, pORF30$_{(\Delta 1114\text{–}1172)}$ still interacted with pORF18 in THS assays. This result was not unexpected, as in this mutant the region corresponding to 33 C-terminal residues is still present. However, a significantly lower level of β-Gal expression in comparison with that of yeast cells expressing full-length pORF30 plus pORF18 was observed. Thus, to examine the specific contribution of residues 1114 to 1172 to pORF18 binding, the ability of this fragment [pORF30$_{(\Delta 1\text{–}1113,\ 1173\text{–}1220)}$] to interact with the accessory protein was tested. We found that the pORF30$_{(\Delta 1\text{–}1113,\ 1173\text{–}1220)}$ mutant could bind pORF18 in two-hybrid assays.

Finally, as it was recently shown that the last two residues of some herpesvirus DNA polymerase catalytic subunits play a role in the interaction with the cognate accessory protein (33, 37, 65), we asked whether the very last amino acids of pORF30 could also be important for pORF18 binding. At the extreme C terminus, the pORF30 protein presents a proline followed by the three residues, HQS, which are conserved in VZV and BHV-1 but not in other alphaherpesviruses (e.g., HSV-1 and PRV) (3). We found that deleting the three C-terminal residues of pORF30 significantly impaired its ability to interact with the accessory protein (Table 2).

Taken together, these results demonstrate that pORF30 physically interacts with pORF18 through both a C-proximal region and the extreme C terminus.

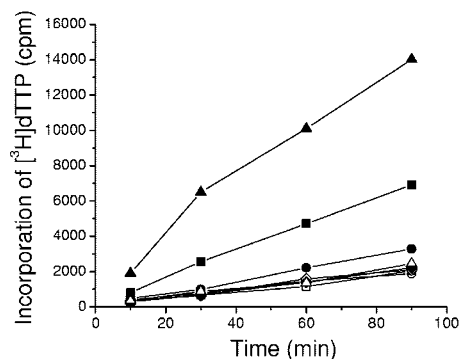

FIG. 9. Stimulation of DNA polymerase activity of pORF30 C-proximal and C-terminal mutants by pORF18. The DNA synthesis activities of in vitro-transcribed-translated pORF30 deletion mutants in the absence and in the presence of in vitro-expressed pORF18 were analyzed in a DNA polymerase assay by measuring the incorporation of [^{3}H]dTTP into a poly(dA)-oligo(dT) template. △, pORF30 alone; ▲, pORF30 plus pORF18; □, pORF30$_{(\Delta 1114-1172)}$ alone; ■, pORF30$_{(\Delta 1114-1172)}$ plus pORF18; ○, pORF30$_{(\Delta 1188-1220)}$ alone; ●, pORF30$_{(\Delta 1188-1220)}$ plus pORF18; ◇, pORF30$_{(\Delta 1114-1220)}$ alone; ◆, pORF30$_{(\Delta 1114-1220)}$ plus pORF18.

Both a C-proximal region and the extreme C terminus of pORF30 are important for its functional interaction with pORF18. In another approach to assess the importance of the C-proximal and C-terminal regions of pORF30 in its interaction with pORF18, we tested the ability of pORF30 mutants wherein these regions were deleted to be stimulated by pORF18. The DNA polymerase activity of in vitro transcription-translation products of *ORF30* deletion mutants lacking the C-proximal domain, the very C-terminal domain, or both [pORF30$_{(\Delta 1114-1172)}$, pORF30$_{(\Delta 1188-1220)}$, and pORF30$_{(\Delta 1114-1220)}$, respectively] was assayed in the absence and in the presence of in vitro-expressed pORF18 and compared to that of wild-type pORF30. Similar amounts of each pORF30 mutant, as assessed by SDS-PAGE and autoradiography (data not shown), were used in each experiment. In these assays, the pORF30$_{(\Delta 1188-1220)}$ mutant possessed DNA polymerase activity similar to that of full-length protein in the absence of the accessory protein, and yet the addition of pORF18 almost failed to enhance its activity (Fig. 9). These results demonstrated that the 33 C-terminal residues of pORF30 are not necessary for basal activity but are required for functional interaction with pORF18.

Similar results were obtained with pORF30$_{(\Delta 1114-1172)}$, as this mutant also exhibited basal DNA polymerase activity similar to that of the wild-type protein but was not stimulated by pORF18 as efficiently as full-length pORF30 (Fig. 9). Thus, the C-proximal domain is also not essential for pORF30 catalytic activity but appears to be important for the pORF18-mediated stimulation of DNA polymerase activity. Consistent with its complete inability to bind pORF18 (Table 2), the pORF30 mutant lacking both the C-proximal and the very C-terminal domains [pORF30$_{(\Delta 1114-1220)}$] failed to be stimulated by pORF18, although it retained basal activity equivalent to that of full-length pORF30 (Fig. 9). To ensure that the inability of the pORF30 mutants to be stimulated by pORF18 was not due to instability of the mutant polypeptides, aliquots of the Pol assays were analyzed by SDS-PAGE after DNA synthesis had been terminated, but no degradation of the in vitro-expressed polypeptides was detected (data not shown).

The locations of the pORF30 mutations and the effect they had on pORF30-pORF18 complex formation and on stimulation of pORF30 activity by pORF18 are summarized in Fig. 8. The pORF30 deletions affecting pORF18 binding are all located at the C terminus of the protein, while deletions upstream of the 107 C-terminal residues have no consistent effect. Therefore, the C terminus of the polypeptide is important for pORF30-pORF18 interaction. Within this region, both a conserved domain corresponding to aa 1114 to 1172 and the last 33 residues of pORF30 play a role in physical and functional interaction with pORF18, as deletion of each of these fragments impaired both binding to, and stimulation by, pORF18. Moreover, both the C-proximal domain and the extreme C terminus of pORF30 are necessary and sufficient to bind pORF18. The inhibitory effect of deletion of the pORF30 C-proximal and C-terminal domains on stimulation of DNA polymerase activity by pORF18 correlates with the effect of each deletion on binding of the mutants to pORF18, as detected by THS assays. This correlation strongly suggests that the role of these segments of pORF30 in stimulation by pORF18 is via physical interaction with pORF18.

DISCUSSION

This is, to our knowledge, the first study reporting the expression and functional characterization of EHV-1 DNA polymerase. Indeed, after the initial identification by Telford et al. (56) of two EHV-1 ORFs, *ORF30* and *ORF18*, with significant homology to the HSV-1 *UL30* and *UL42* genes, respectively, no study of these ORFs has been reported. Here we demonstrate that the product of the EHV-1 gene (*ORF30*), encoding the putative DNA polymerase catalytic subunit, possesses DNA synthesis activity in the absence of the Pol accessory protein (pORF18) and that the *ORF18*-encoded protein can physically interact with pORF30 and stimulate both nucleotide incorporation and long-chain DNA synthesis by pORF30. Thus, the EHV-1 pORF30 and pORF18 proteins provide functions similar to those of their HSV-1 counterparts. However, despite these similarities, the EHV-1 and HSV-1 proteins cannot physically or functionally substitute for each other.

Like HSV-1 DNA polymerase, which is a two-subunit complex (12, 23), the formation of the pORF30-pORF18 complex does not require the participation of any other virus-encoded protein. The stoichiometry of the two subunits in EHV-1 DNA polymerase is probably 1:1, as neither pORF30 nor pORF18 could form homodimers in THS assays and maximal stimulation of pORF30 activity by pORF18 was observed at an equimolar ratio of the two proteins. Although the stoichiometry of pORF30 and pORF18 in the native EHV-1 holoenzyme could be different, we think this is most unlikely, since only accessory subunits of beta- and gammaherpesvirus Pols have been shown to form homodimers (2, 9), whereas the counterpart of an alphaherpesvirus DNA polymerase, i.e., HSV-1 UL42, is a monomer both in solution and in the crystal structure (48, 65). However, further biochemical and structural studies are needed to test this speculation.

Effects of salt concentration on pORF30 and pORF30-pORF18 activity. One of the features common to all studied

herpesvirus DNA polymerases is the stimulation of their activity in buffers with high salt concentrations (11). The DNA polymerase activity of pORF30 and of pORF30-pORF18 was also found to be dependent on the salt concentration. The pORF30 protein alone exhibited a sharp salt optimum at 50 mM KCl. In this respect, the behavior of EHV-1 Pol is very similar to that of HSV-1 UL30 (25), while it differs from that of other herpesvirus DNA polymerase catalytic subunits, where maximum activity occurs either in the absence of salt (PRV and EBV Pols [3, 59]) or in the presence of salt concentrations higher than 50 mM (HCMV Pol [10, 61]). In contrast, the salt-dependent profile of DNA polymerase activity of the pORF30-pORF18 complex appears to be different from that of all other herpesvirus DNA polymerases. Specifically, the EHV-1 holoenzyme exhibited maximum activity in the absence of salt, while for the HSV-1, PRV, HCMV, and EBV counterparts little or almost no activity was observed in the absence of salt and increasing activity was detected when increasing amounts of salt were added (3, 10, 25, 59, 61). Irrespective of these differences, the net effect is that the EHV-1 Pol accessory protein inhibits the activity of the catalytic subunit at low salt concentrations and stimulates its activity in the presence of high salt concentrations, as reported for HSV-1, PRV, HCMV, and EBV DNA polymerase (3, 10, 25, 59, 61).

The pORF30 protein interacts with pORF18 through the extreme C terminus. In this work, regions in pORF30 important for both physical and functional interaction with pORF18 were identified. By extensive mutational analysis, we have demonstrated that the sequences of pORF30 important for pORF18 binding reside in the C terminus. In fact, a pORF30 mutant lacking last 107 amino acids failed to be stimulated by, or to form complexes with, pORF18. Moreover, the 107 C-terminal residues of pORF30 are both necessary and sufficient for interacting with pORF18, as a mutant that expresses only this fragment could interact with pORF18 in a manner similar to that of the full-length protein.

The C terminus of several other herpesvirus Pols is also required for interaction with, and stimulation by, the accessory protein but not for basal polymerase activity (3, 14, 17, 33, 37, 39, 54, 57). In HSV-1, both biophysical and crystallographic studies demonstrated that the 18 C-terminal residues of UL30 are sufficient to bind UL42 (8, 65), although possible contributions by regions upstream have not been excluded (see below). Similarly, a region of HCMV Pol corresponding to the last 22 residues was recently shown to be both necessary and sufficient for UL44 binding (33) and deletion of the C-terminal 30 residues of PRV Pol abolished its ability to be stimulated by its accessory protein (3).

These observations suggest a converging theme in the interaction between the two subunits of herpesvirus DNA polymerases, wherein the extreme C terminus of the catalytic subunit is crucial for binding to the cognate accessory protein. Thus, the role of the extreme C terminus of pORF30 in pORF18 binding was investigated. Our results demonstrate that a region corresponding to the 33 C-terminal residues of pORF30 is important for physical and functional interaction with pORF18. Furthermore, within the C-terminal region the last three amino acids of pORF30 seem to play a role in pORF30-pORF18 interaction, as deletion of these residues impaired the ability of pORF30 to bind pORF18 and also slightly impaired its ability to be stimulated by pORF18 without affecting basal DNA polymerase activity (data not shown). Analogously, deletion of the two C-terminal cysteines of the HCMV DNA polymerase catalytic subunit, UL54, also reduced interactions with UL44 (33). The structural basis for the role of the very C-terminal residues of pORF30 in pORF18 binding remains to be determined.

A highly conserved C-proximal region of pORF30 also has a role in pORF18 binding. Computer analysis of EHV-1 pORF30 using homology searches showed that in addition to the eight regions of homology (I through VII and A) observed among a variety of prokaryotic, eukaryotic, and viral DNA polymerases (5, 26, 60, 62), additional regions of sequence homology can be identified among the herpesvirus Pol genes (data not shown). Alignment of several alphaherpesvirus DNA polymerase catalytic subunits (i.e., those of HSV-1, VZV, PRV, BHV-1, and EHV-1) revealed 36.7% sequence identity within the entire pORF30 protein. A similar level of homology (31.8% identity) was observed across the 107-aa C-terminal pORF18-binding domain; however, within this region, a C-proximal domain which is more highly conserved (76.3% identity) could be recognized between residues 1114 and 1172. Interestingly, only two residues (6%) of the extreme C terminus (aa 1173 to 1220) are identical.

While the highly conserved regions in the central portion of herpesvirus Pols have been implicated in substrate binding and catalytic activity (11), the functional significance of the C-proximal conserved domain has not yet been determined. In HSV-1, several observations have suggested that there may be a contribution of residues upstream of the extreme C terminus of UL30 to the interaction with UL42. First, deletions within the region upstream of the C-terminal 36 residues of UL30 partially affected binding to UL42 in coimmunoprecipitation experiments (14). Second, a truncated UL30 mutant lacking the C-terminal 27 residues bound UL42 fourfold less well than the full-length protein, suggesting that these amino acids contribute only 75% of the binding (39). However, direct evidence for a role for these upstream regions is lacking.

The results presented here clearly implicate residues between aa 1114 and 1220 of pORF30 in productive interaction with pORF18. First, deletion of this region significantly affected both the physical and functional associations. Second, this fragment alone was able to bind pORF18. Although the highly conserved C-proximal domain of pORF30 appears to play a role in pORF30-pORF18 binding, the EHV-1 DNA polymerase subunits could not be physically or functionally replaced by their HSV-1 counterparts and the C-proximal fragment could not interact with UL42 in two-hybrid assays (data not shown). These observations suggest that the interaction between pORF30 and pORF18 through the C-proximal region likely involves nonconserved residues. Site-directed mutagenesis of this region of pORF30 will be necessary to test this hypothesis.

Implications for drug discovery and for studies of EHV-1 pathogenesis. Overall, it appears that both the C-proximal domain and the extreme C terminus of pORF30 contribute to a productive interaction with pORF18. In the future, the identification of the residues within these regions of EHV-1 Pol that are crucial for binding to the accessory protein may help in the design of antiviral agents that inhibit EHV-1 replication

by disruption of the pORF30-pORF18 interaction (35, 36). Encouragement for this approach comes from the recent identification of small-molecule compounds that inhibit HSV-1 (46) or HCMV (34) DNA polymerase subunit interactions and virus replication in cell culture.

More generally, the studies reported here may also contribute to understanding of the role of pORF30 and pORF18 in EHV-1 pathogenesis. Notably, recent studies have demonstrated that pORF30 sequence variation is associated with pathogenic potential, as an *ORF30* single-nucleotide polymorphism (G/A_{2254}), corresponding to amino acid variation D/N_{752} of pORF30, shows a very strong association with neuropathogenic/non-neuropathogenic EHV-1 isolates (N. Davis-Poynter et al., Abstr. 30th Int. Herpesvir. Worksh., abstr. 11.5, 2005). Thus, it has been speculated that the D/N_{752} coding change may affect functional properties of the viral DNA polymerase that have a role in the etiology of EHV-1 neurological disease. The biochemical studies and assays described herein may offer a rapid and facile means for testing such a prediction.

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