JOURNAL OF VIROLOGY, June 1994, p. 3674–3681
0022-538X/94/$04.00+0

Vol. 68, No. 6

Cloning and Expression of an Equine Herpesvirus 1 Origin-Binding Protein

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Received 23 November 1993/Accepted 26 February 1994

Equine herpesvirus 1 (EHV-1) is an important pathogen of horses and is closely related to several important human pathogens, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus. The EHV-1 genome contains open reading frames similar in sequence to the HSV-1 replication genes. PCR was used to clone EHV-1 gene 53, which is similar in sequence to the HSV-1 UL9 gene. The gene 53 product has regions of striking similarity to the HSV-1 UL9 and VZV gene 51 products. In vitro transcription and translation of this gene generated a protein of 87 kDa as measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Further characterization of this protein was accomplished through the use of gel shift analysis. The in vitro-synthesized protein bound sequence specifically to EHV-1 Ori_S as well as HSV-1 Ori_S. A series of triple-base-pair substitutions across the previously defined HSV-1 origin-binding protein consensus site was used in gel shift analysis to show that the EHV-1 origin-binding protein bound to the same consensus site as the HSV-1 origin-binding protein, 5′-CGTTCGCACTT-3′. Using a nuclear extract of EHV-1-infected RK13 cells, we have identified an activity that interacts similarly with this consensus site. In gel shift assays, the retarded band arising from the nuclear extract migrated similarly to the retarded band arising from in vitro-translated EHV-1 gene 53. An N-terminal deletion of EHV-1 gene 53 was also created, expressed in vitro, and used in gel shift assays to localize the DNA-binding domain. Results of these experiments indicated that amino acids 1 to 499 were dispensable for binding and that the C-terminal fragment (amino acids 500 to 888) recognized the same consensus site as did the wild-type protein. Thus, the product of EHV-1 gene 53 is an origin-binding protein with a high degree of similarity to the HSV-1 and varicella-zoster virus origin-binding proteins and possibly serves as the initiator of DNA replication in EHV-1.

Equine herpesvirus 1 (EHV-1) is a member of the subfamily *Alphaherpesvirinae* (19). As a member of this group, EHV-1 is closely related to several important human pathogens including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV) (17, 21). EHV-1 is also an important pathogen of horses and can cause neurological disorders, respiratory infection, and epizootic abortions in pregnant mares (1). A better understanding of EHV-1 biology may contribute significantly to the understanding of other alphaherpesviruses. Specifically, analysis of DNA replication in EHV-1 is important for several reasons. A better understanding of the replication process may lead to better management of the disease in horses. In addition, an understanding of the mechanisms of DNA replication in EHV-1 may help elucidate the mechanisms of replication of the closely related human herpesviruses, which may in turn provide a means of intervention in the diseases caused by these viruses. Third, knowledge of EHV-1 DNA replication would contribute toward the generation of a general model of DNA replication for the alphaherpesviruses.

A *cis*-acting sequence in the EHV-1 genome that functions as an origin of DNA replication has been identified (2). Two copies of this sequence, designated Ori_S, are present in the wild-type genome, one each in the two terminal repeats, IR_S and TR_S. The EHV-1 Ori_S contains three copies of a sequence found in all alphaherpesvirus lytic origins, 5′-CGTTCGCAC-3′. This sequence is required for binding of the HSV-1 and VZV initiator proteins (4, 8, 14, 20, 23). EHV-1 Ori_S also contains a prominent AT-rich stretch. No other viral replication origins have been functionally identified in EHV-1. However, the viral genome contains a region similar to HSV-1 Ori_L (18). No *trans*-acting replication factors have yet been functionally identified. Identification of such factors will be critical for an understanding of EHV-1 DNA replication.

The objective of this study was to identify the EHV-1 origin-binding protein (OBP). PCR was used to clone EHV-1 gene 53, and the construct was used to express the gene 53 product in vitro. Labeled EHV Ori_S and the in vitro-synthesized protein were used in gel shift experiments to show that this protein binds specifically to EHV-1 Ori_S. The product of EHV-1 gene 53, which will be referred to as the EHV-1 OBP, also bound specifically to a truncated HSV-1 Ori_S and recognized a consensus binding site similar to that recognized by the HSV-1 OBP. A deletion of the EHV-1 OBP was constructed and used to demonstrate that the DNA-binding domain resides in the C-terminal half of the protein. Taken together, the results of these experiments indicate that the product of EHV-1 gene 53 is an OBP with a high degree of similarity to the HSV-1 and VZV OBPs. Therefore, EHV gene 53, HSV-1 UL9, and VZV gene 51 encode similar proteins that likely perform key roles in the initiation of DNA replication in their respective systems.

MATERIALS AND METHODS

Cells and viruses. EHV-1, Kentucky A strain, was the kind gift of Dennis O'Callaghan. RK13 cells were acquired from the American Type Culture Collection, Rockville, Md. Cells were

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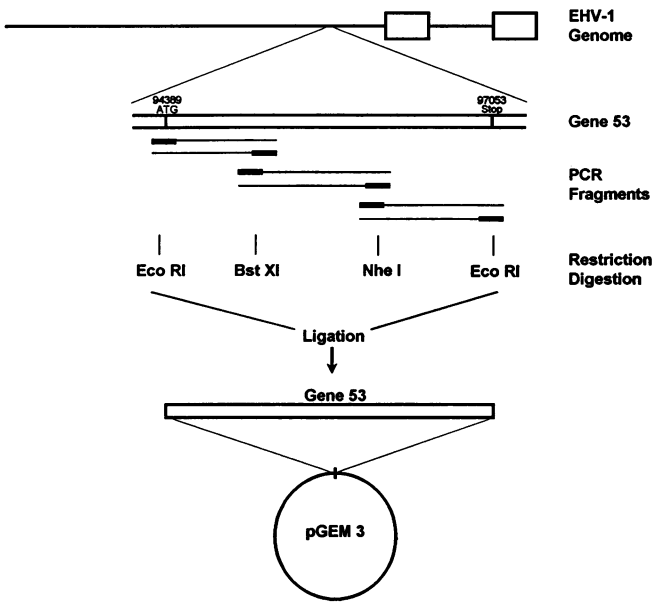

FIG. 1. Cloning of EHV-1 gene 53. Three sets of overlapping fragments encompassing the entire gene 53 were prepared by PCR as described in Materials and Methods. These fragments were restricted to generate complementary ends and ligated into the *Eco*RI site of pGEM3 to reconstruct the wild-type gene. The schematic shows the orientation of gene 53 relative to the EHV-1 genome, the resultant PCR fragments, the restriction enzymes used, and the final ligation into pGEM3. The boxes in the top line represent repeated sequences in the EHV-1 genome.

grown in minimal essential medium supplemented with 10% fetal calf serum.

Isolation of viral DNA. Confluent RK13 cells in 150-cm^2 flasks were infected at a multiplicity of infection of 0.01. After 48 h, the supernatant was removed and spun at 3,000 rpm (800 $\times$ *g*) for 5 min at 4°C to pellet any cells. The virus was then precipitated from the supernatant with 7% (wt/vol) polyethylene glycol 6000 and 2.3% (wt/vol) sodium chloride (NaCl). This mixture was stirred overnight at 4°C and pelleted by spinning at 8,500 rpm (11,790 $\times$ *g*) for 1 h at 4°C. The pellet was washed two times in 0.001 M phosphate buffer and dialyzed overnight at 4°C against the same buffer. After dialysis, the solution was treated with 10% SDS–10 μg of proteinase K per ml in TE (10 mM Tris, 1 mM EDTA, pH 8.0) and incubated at 55°C for 4 h. This was followed by phenol-chloroform extraction and ethanol precipitation. The DNA was finally resuspended in TE.

Cloning and expression of EHV-1 gene 53. The previously published sequence for a British EHV-1 isolate (21) was used to design a series of PCR primers. A schematic of the cloning scheme is shown in Fig. 1. The following primers were used in the synthesis of the respective fragments: primer 1, 5′-CGGGTCGAATTCGAAGCC-3′, and primer 2, 5′-CTGAA ATGGGTCTGGAATCCG-3′, to generate the fragment from nucleotide position 94389 to 95322; primer 3, 5′-GAACTG ACGTATTGCGGGCAG-3′, and primer 4, 5′-CCTTTTC ATAGCGGGACCCTG-3′, to generate the fragment from nucleotide position 95230 to 96670; and primer 5, 5′-CC GGGCTGGACATGTCGGAAG-3′, and primer 6, 5′-GCCC AGCTTAAGCTTCGGCCATTCGACGCTGCCC-3′, to generate the fragment from position 96482 to 97052. Fragments were generated by a standard PCR protocol (Cetus) and *Taq* DNA polymerase (Promega), purified by low-melting-point agarose gel electrophoresis, and digested with the following enzymes: fragment 1, *Eco*RI and *Bst*XI; fragment 2, *Bst*XI and *Nhe*I; and fragment 3, *Nhe*I and *Eco*RI. Each restriction fragment was ligated at an equimolar ratio into the *Eco*RI site of pGEM3 (Promega), and the ligation mixture was used to transform DH5. Colonies were selected in the presence of ampicillin. Plasmid DNA was isolated from these colonies and screened by restriction analysis. Expression was tested with the TNT lysate system (Promega). A positive clone was then sequenced in its entirety with Sequenase version 2.0 (United States Biochemical) to confirm its relationship to the previously published sequence of EHV-1 gene 53 (21). Proteins for use in DNA-binding assays were expressed in vitro with the TNT system (Promega).

The molecular weight of the translated product was determined by comparing the migration rate of the ^{35}S-labeled protein with the migration rate of a set of high-range molecular weight numbers (GIBCO-BRL) that were stained with Coomassie blue after they were run in parallel with the ^{35}S-labeled material. Comparison of the amino acid sequence of the EHV-1 gene 53 product with those of the HSV-1 and VZV OBPs was done with software from DNAStar (Madison, Wis.).

PCR was used to create an N-terminal deletion mutant of gene 53. A deletion of the N-terminal 499 amino acids, del 1–499, was created by introducing an ATG at the equivalent nucleotide position, 95883. PCR fragments were filled in with DNA polymerase I large fragment (Klenow) ligated into the *Sma*I site of pGEM3 and transformed into *Escherichia coli* JM109, and white colonies were selected in the presence of IPTG (isopropyl-β-D thiogalactopyranoside)–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and ampicillin. Plasmid DNA was isolated and screened by restriction analysis. Clones also were screened for the ability to express the desired product in vitro with the TNT lysate (Promega).

Preparation of nuclear extracts. RK13 cells were infected with EHV-1 Kentucky A strain (or mock infected). Cells were harvested 20 h later, and nuclear extracts were prepared essentially as described by Elias et al. (8), with the exception that sucrose was omitted from the lysis buffer and the final dialysis was done in 10% glycerol. Quantities of protein from both infected and mock-infected extracts were measured with Bio-Rad protein assay reagent as described by the manufacturers. The A_{580} was determined with a Milton Roy Epectronic 201. Absorbance values were plotted against a standard curve generated with bovine serum albumin under similar conditions. To directly analyze protein levels, aliquots of infected and mock-infected extracts were also run on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels and stained with Coomassie blue.

DNA-binding studies. The EHV-1 Ori$_S$ probe used for DNA-binding studies was excised from the plasmid pl-133 (1) (a gift of Dennis O'Callaghan) with *Cla*I and *Spe*I. The fragment was end labeled with [^{32}P]dCTP. The generation of HSV-1 Ori$_{S-1}$ and its triple base substitution mutants was described previously (4, 6). These probes were excised from the parent plasmid by *Eco*RI and *Hin*dIII digestion and labeled with [^{32}P]dCTP.

Gel shift assays were performed under the buffer conditions previously described (5, 15). The reaction mixture contained, in a final volume of 50 μl, 1.5 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia) and 5 μg of sheared calf thymus DNA (Sigma). Reactions were incubated for 30 min at room temperature, after which loading dye was added. Samples were run in 5% 0.5× Tris-borate-EDTA (TBE) polyacrylamide gels at room

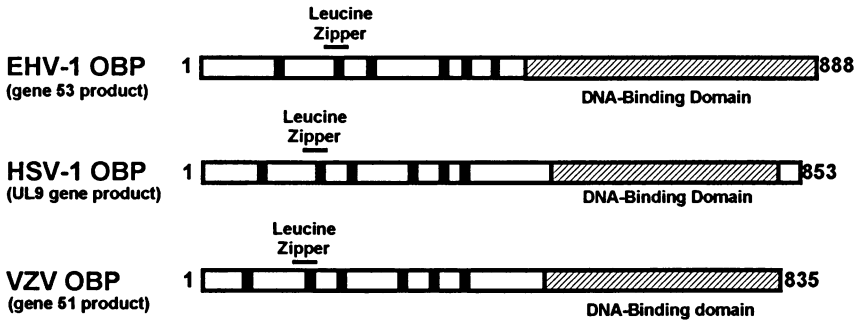

FIG. 2. Comparison of EHV-1, HSV-1, and VZV OBPs. Solid black boxes indicate conserved helicase motifs. The hatched box represents the DNA-binding domains. The position of a leucine zipper is also shown. Note the conservation of these features between the proteins.

temperature. Gels were fixed in glycerol-acetic acid, dried, and exposed for autoradiography. DNA-binding studies with nuclear extracts made use of 5 mg of infected or mock-infected total protein. Binding was done under buffer conditions as described above (15). Reaction mixtures were incubated for 30 min on ice and subsequently run at room temperature as described above.

RESULTS

Cloning and expression of EHV-1 gene 53. Identification and characterization of an EHV-1 OBP is important for better understanding of the mechanism of initiation of DNA replication by this virus and of alphaherpesvirus DNA replication in general. The presence of the conserved sequence 5′-CGTT CGCAC-3′ (within EHV-1 Ori_S) that was previously shown to bind the HSV-1 and VZV OBPs in their respective origins initially suggested that similar protein elements may mediate binding to similar DNA sequences. Telford et al. (21) reported the entire sequence of the EHV-1 genome and identified gene 53 as having a strong sequence similarity to HSV UL9. This allowed us to clone EHV-1 gene 53 by PCR. The steps by which this was accomplished are described in Materials and Methods and are illustrated in Fig. 1. The cloned DNA was completely sequenced to verify its identity. Sequence analysis of the open reading frame revealed several interesting features. First, a series of motifs that are conserved across a superfamily of DNA and RNA helicases was present in the amino-terminal half of the protein. A potential leucine zipper was detected between amino acids 175 and 195 and was similar to that seen in the HSV-1 OBP. EHV-1 gene 53 also had a high degree of sequence similarity to the C-terminal halves of the HSV-1 UL9 gene and VZV gene 51. These HSV-1 and VZV genes encode proteins that bind sequence specifically to binding sites within their respective origins of DNA replication. The results of the comparison with HSV-1 and VZV OBPs are shown in Fig. 2.

Regions of the EHV-1 gene 53 product that had at least a 50% sequence similarity to these proteins are shown. It is clear from this analysis that the product of EHV-1 gene 53 has a striking similarity to the OBPs from VZV and HSV-1. Regions of similarity are found throughout the length of the EHV-1 gene 53 product. The regions of similarity with the HSV-1 and VZV OBPs clearly overlap, indicating the apparent conservation of functional domains. In order to generate protein for DNA-binding assays, the wild-type gene 53 construct was used to express the protein in vitro with the TNT system (Promega) as described in Materials and Methods. An aliquot of the ^{35}S-labeled protein was run on an SDS–10% polyacrylamide gel. The results are shown in Fig. 3. A single band in the wild-type lane indicated the expression of the wild-type gene 53 product. This band migrated at a position consistent with a 97-kDa protein. No protein was visible in the mock-extract lane. This result clearly indicates the successful expression of the wild-type gene 53 product.

Binding of wild-type gene 53 product to an EHV-1 origin of DNA replication. In order to test the binding of the wild-type gene 53 product, gel shift assays were performed with a previously defined EHV-1 origin of DNA replication, Ori_S (2). The sequence of this origin is shown in Fig. 4. EHV-1 Ori_S contains three sites with a high degree of similarity to OBP-binding sites in HSV-1 and VZV (4, 14). EHV-1 Ori_S was excised from the parent plasmid and end labeled with [^{32}P]dCTP. This labeled DNA was used as a probe in gel shift assays with in vitro-expressed gene 53 product as described in Materials and Methods. Figure 4 shows the result of such an assay. In the presence of wild-type gene 53 product, three complexes (complexes I, II, and III) were formed with EHV-1 Ori_S (Fig. 4, lane 3). These complexes could be the result of the synthetic gene 53 protein binding to each of three presumptive OBP-binding sites. Alternatively, the smeared nature of the upper complex may indicate the formation of a higher-order structure that did not clearly resolve under these conditions. This was in contrast to the migration of the free probe (Fig. 4, lane 1) and the lack of complex formation when a mock-programmed extract was used (lane 2). These complexes were

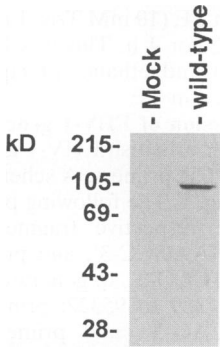

FIG. 3. Expression of EHV-1 gene 53. EHV-1 gene 53 was expressed with the TNT lysate system (Promega). An aliquot of the ^{35}S-labeled protein was run on an SDS–10% polyacrylamide gel and visualized by autoradiography. Note the presence of a single band relative to the position of the molecular mass marker. Note also the absence of any complex in the mock-programmed lane.

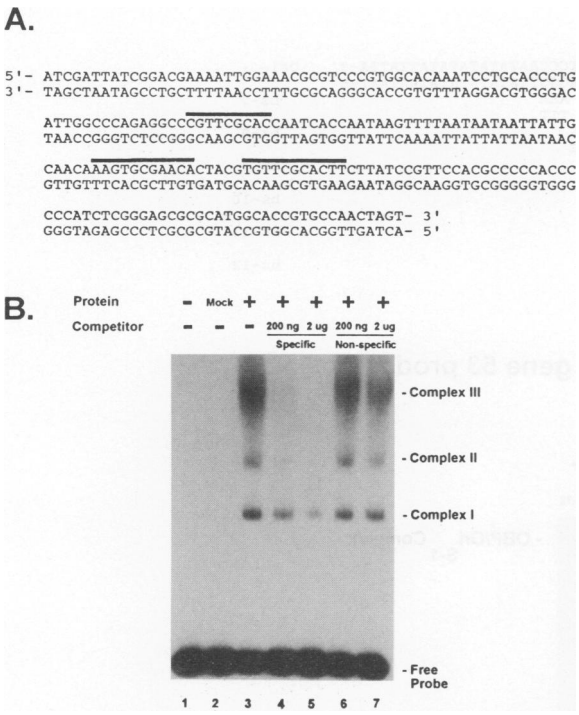

FIG. 4. Binding of the EHV-1 gene 53 product to EHV-1 Ori_S. (A) Sequence of EHV-1 Ori_S. Heavy lines indicate DNA sequences similar to HSV-1 and VZV sequences that interact with their respective OBPs. (B) Binding of gene 53 product. Gel shift analysis of gene 53 product on EHV-1 Ori_S was performed as described in Materials and Methods. Note the formation of three complexes (I, II, and III) relative to the free probe.

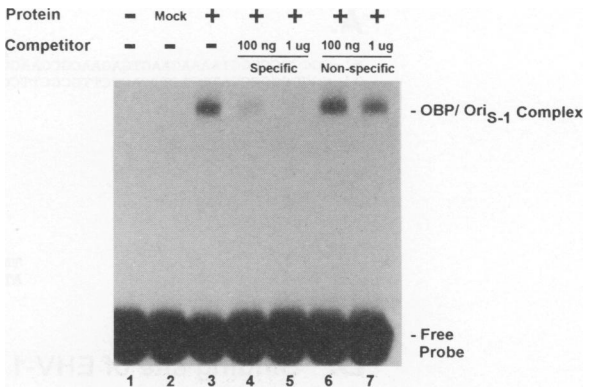

FIG. 5. Binding of EHV-1 gene 53 to HSV-1 Ori_{S-1}. In vitro-synthesized EHV-1 gene 53 and radiolabeled HSV-1 Ori_{S-1} were used in gel shift analysis as described in the text. Note the formation of a single complex and the inhibition of this complex by specific competitor but not by nonspecific competitor (compare lanes 4 and 5 with lanes 6 and 7).

tested for their specificity with specific and nonspecific competitors. Addition of EHV-1 Ori_S-containing specific competitor (pl-120, containing EHV-1 Ori_S [2]) clearly had a drastic effect on complex formation, as complexes II and III were eliminated while complex I was reduced dramatically. Addition of nonspecific competitor (pBR322) had little effect on complex formation (Fig. 4B; compare lanes 4 and 5 with lanes 6 and 7). Thus, EHV-1 gene 53 encodes a protein that bound specifically to EHV-1 Ori_S. Therefore, this product is herein referred to as the EHV-1 OBP.

Binding of the EHV-1 OBP to HSV-1 Ori_{S-1}. The high degree of similarity between alphaherpesvirus OBPs and the conservation of OBP-binding sites on the respective origins of DNA replication imply that the EHV-1 OBP may bind to the HSV-1 origin. To test this, EHV-1 OBP was used in a gel shift assay with the previously described Ori_{S-1} template (6). Ori_{S-1} contains a single OBP-binding site. Incubation of the EHV-1 OBP with HSV-1 Ori_{S-1} resulted in the formation of a single complex (Fig. 5, lane 3). This single complex presumably resulted from the binding of a single EHV-1 OBP to the lone binding site on Ori_{S-1}. No complex was formed when mock extract was used (Fig. 5, lane 2). The complex between EHV-1 OBP and Ori_{S-1} was specific, as it was inhibited by specific competitor (pl-120, containing EHV-1 Ori_S [2]) (Fig. 5, lanes 4 and 5) but not by nonspecific competitor (pBR322) (lanes 6 and 7). Thus, EHV-1 OBP forms a specific complex with HSV-1 Ori_{S-1}.

Determination of the DNA-binding site of EHV-1 OBP. The specific binding of EHV-1 OBP to HSV-1 Ori_{S-1} provides the basis for determining the binding site recognized by this protein. A set of base substitution mutants across the OBP-binding site on Ori_{S-1} were used previously to determine the HSV-1 OBP-binding site (4). These constructs were excised from the parent plasmid and end labeled with [^{32}P]dCTP and the Klenow fragment of *E. coli* DNA polymerase I. The resulting probes were used with in vitro-expressed EHV-1 OBP in gel shift analysis. The nature of the Ori_{S-1} mutations is shown in Fig. 6A. Figure 6B shows the result of the gel shift experiment. A single complex was formed between EHV-1 OBP and Ori_{S-1} (Fig. 6B, lane 3). Triple base substitution mutants bs-7 and bs-8 also formed wild-type levels of complex (Fig. 6B, lanes 4 and 5). However, mutations bs-9, bs-10, and bs-11 resulted in a loss of complex formation. Mutant bs-12 showed only a faint level of complex formation. These results indicated that the EHV-1 OBP recognized the sequence 5′-GCGTTCGCACTT-3′. This is essentially the same sequence that is critical for binding of the HSV-1 OBP, 5′-CGTTCGCACTT-3′ (4, 14).

Construction, expression, and DNA binding of an EHV-1 OBP deletion mutant. Previous analyses of the DNA-binding domain of the OBPs of HSV-1 and VZV (5, 20, 22) and the sequence similarities of the product of gene 53 of EHV-1 with the OBPs of HSV-1 and VZV led us to speculate that the DNA-binding domain of the EHV-1 OBP might be located on the C-terminal half of the molecule. Therefore, in order to determine which region(s) of the EHV-1 OBP are required for sequence-specific DNA binding, an N-terminal deletion mutant of the EHV-1 OBP was constructed as described in Materials and Methods. This deleted gene was expressed in vitro with the TNT system (Promega). An aliquot of the ^{35}S-labeled protein was run in an SDS–15% polyacrylamide gel and visualized by autoradiography. Figure 7B shows the results of this expression. As expected, no protein was generated in the mock extract. Expression of del 1–499 generated a major product of the predicted size. A second, smaller product was present and probably resulted from internal initiation downstream at an in-frame start codon. In order to determine the ability of the EHV-1 OBP deletion mutant to bind DNA, gel shift assays were performed with in vitro-expressed protein and EHV-1 Ori_S as a probe. Equimolar amounts of wild-type and mutant protein (determined by trichloroacetic acid precipitation, as specified in the Promega protocol) were incubated with the EHV-1 Ori_S probe as described in Materials and Methods.

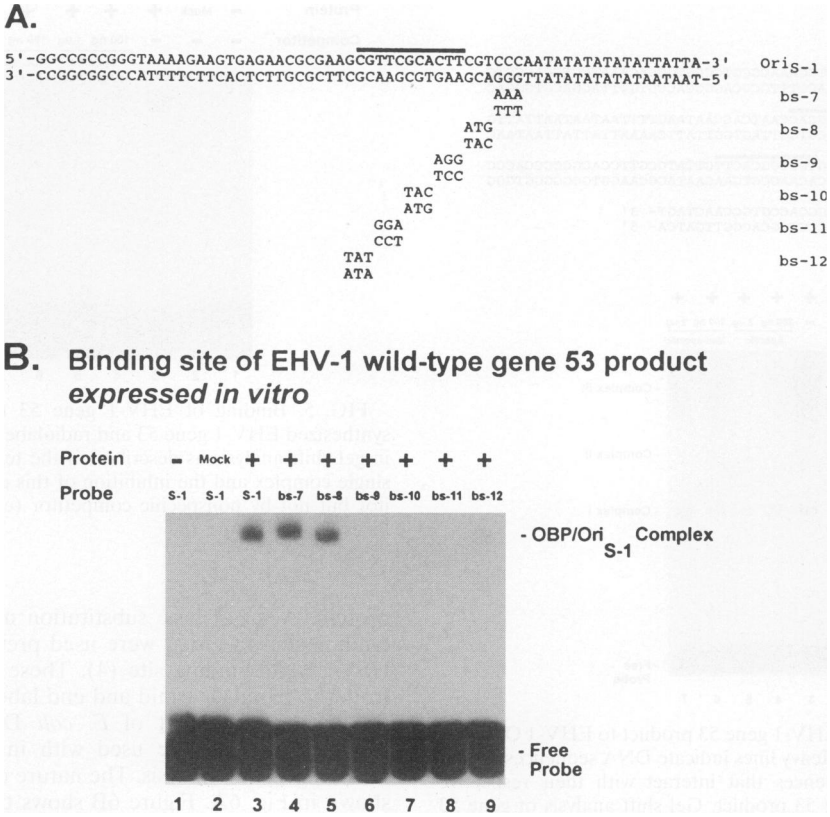

FIG. 6. EHV-1 gene 53 DNA-binding site. (A) Sequence of HSV-1 Ori_{S-1} and triple base substitution mutants. The heavy black line indicates the binding site of HSV-1 OBP. (B) Gel shift analysis. Note complex formation between synthetic EHV-1 gene 53 protein and Ori_{S-1} (S-1), bs-7, and bs-8 and the loss of complex formation with bs-9, bs-10, bs-11, and bs-12.

Figure 7C shows the results of this binding. Wild-type OBP generated three complexes on EHV-1 Ori_S, as previously seen (Fig. 7C, lane 3). Interestingly, del 1–499 formed one prominent lower complex and two slower-migrating, but faint, complexes. Thus, the amino-terminal 499 amino acids were dispensable for binding.

Determination of the DNA sequence recognized by del 1–499. In order to confirm that the C-terminal DNA-binding domain of EHV-1 OBP was mediating the same sequence-specific binding as the wild-type protein, in vitro-expressed del 1–499 was used to perform gel shift analysis with wild-type and mutant Ori_{S-1} constructs. The locations of the mutations are shown in Fig. 8A. Binding conditions were the same as those described for the wild-type protein. The results illustrated in Fig. 8B clearly demonstrated that del 1–499 formed a single complex with Ori_{S-1} (lane 3). Base substitution mutants bs-7 and bs-8 also formed complexes at wild-type levels. Mutations bs-9, bs-10, bs-11, and bs-12 resulted in a loss of complex formation. This was the same pattern observed for wild-type protein binding and indicated that the sequence-specific DNA-binding domain was contained within the C-terminal half of the protein, from amino acids 500 to 888.

Binding activity from EHV-1-infected extracts. In order to test for an origin-binding activity from EHV-1-infected cells, nuclear extracts were prepared from infected and mock-infected cells as described in Materials and Methods. Equal amounts of infected and mock-infected protein were used to test for the ability to bind to HSV-1 Ori_{S-1}. In one experiment, in vitro-translated EHV-1 gene 53 product was used in gel shift

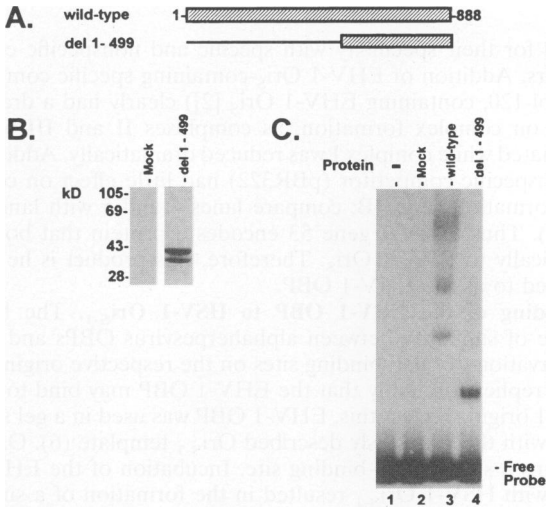

FIG. 7. Expression and binding of an N-terminal EHV-1 gene 53 protein deletion mutant. (A) Scheme of gene 53 protein deletion. The fine line indicates the sequences deleted relative to the wild-type protein. The hatched box represents the area of the protein remaining. (B) Expression of deletion mutant. The mutant was expressed in vitro as described in Materials and Methods. An aliquot of the ^{35}S-labeled protein was run on an SDS–15% polyacrylamide gel and visualized by autoradiography. Note the generation of a product which migrates at the expected rate. No product was seen in a mock-translated lane. (C) DNA binding by the wild-type gene 53 protein and the gene 53 N-terminal deletion mutant. In vitro-expressed protein was used to test binding to EHV Ori_S. Note formation of three complexes in the wild-type lane as well as a complex formed with del 1–499.

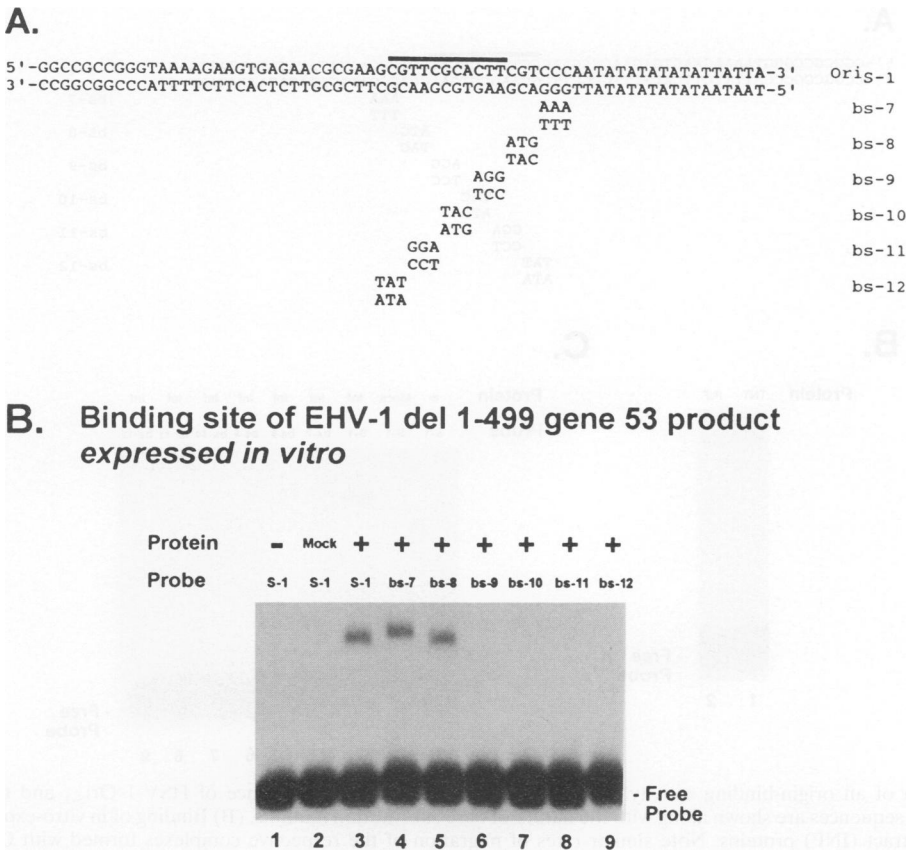

FIG. 8. DNA-binding site of del 1–499. (A) Sequence of HSV-1 Ori$_{S-1}$ and triple base substitution mutants. The heavy line indicates the HSV-1 OBP-binding site. (B) Gel shift analysis with del 1–499. del 1–499 was expressed in vitro and used to perform gel shift analysis with the Ori$_{S-1}$ (S-1) and triple base substitution mutant probes. Note the formation of a single complex with Ori$_{S-1}$, bs-7, and bs-8 as well as the loss of complex formation with bs-9, bs-10, bs-11, and bs-12.

analysis and run in parallel with infected-cell extract. The results of this experiment are shown in Fig. 9B. These results clearly showed that the complexes generated by in vitro-expressed and infected-cell proteins migrated at similar rates. To further test the binding of the infected-cell protein, Ori$_{S-1}$ and base substitution mutants were used as previously described. The results in Fig. 9C clearly show that the infected-cell protein (lanes 3 to 9) recognizes the same DNA sequence that the EHV-1 gene 53 product and the HSV-1 OBP recognize (4). Note the lack of complex formation by mock-infected cell protein (Fig. 9C, lane 2). Thus, EHV-1-infected cells contain an activity similar to the DNA-binding activity of the EHV-1 gene 53 product expressed by in vitro transcription and translation.

DISCUSSION

Telford et al. (21) showed that EHV-1 gene 53 has a strong sequence similarity to HSV-1 UL9 and thus may code for EHV-1 OBP. Data presented in this communication describing the cloning, expression, and analysis of EHV-1 gene 53 verified that prediction. Several critical factors indicated that this gene was a homolog of HSV-1 UL9 and VZV gene 51. First, the product of EHV-1 gene 53 bound sequence specifically to EHV-1 Ori$_S$ (Fig. 4). This binding apparently formed a higher-order structure on the origin, as multiple complexes were formed. In addition, EHV-1 OBP also bound specifically to HSV-1 Ori$_{S-1}$ and recognized the same consensus sequence as the HSV-1 OBP. Finally, the DNA-binding domain determined by us localizes to the region of the protein determined for both the HSV-1 and VZV OBPs. Furthermore, an origin-binding activity is present in infected cells. This activity generates a gel shift complex that migrates at a rate similar to that of the in vitro-expressed gene 53 product and also recognizes the same DNA sequence. In addition to functional implications designating the EHV-1 OBP as the initiator of DNA replication, sequence analysis also revealed a close relationship between EHV-1 gene 53, HSV-1 UL9, and VZV gene 51 (Fig. 2).

Within the amino terminus of these proteins, six domains that are well conserved with a family of DNA and RNA helicases are present (10, 11, 13). The spatial arrangement of these motifs is well conserved among the three proteins. These motifs are required for replication mediated by HSV-1 UL9 (16). In addition, HSV-1 UL9 possesses DNA-dependent ATPase and helicase functions (3). This is consistent with the idea that this protein serves as the initiator of DNA replication in HSV-1. Presumably, these motifs would be required to unwind the helices at the origins to allow accessibility of the DNA template to the replication machinery. Thus, it is not surprising that the EHV-1 OBP would possess these motifs, since their function would be consistent with the protein's predicted role in initiation of DNA replication. Another conserved motif of interest is a leucine zipper that overlaps the

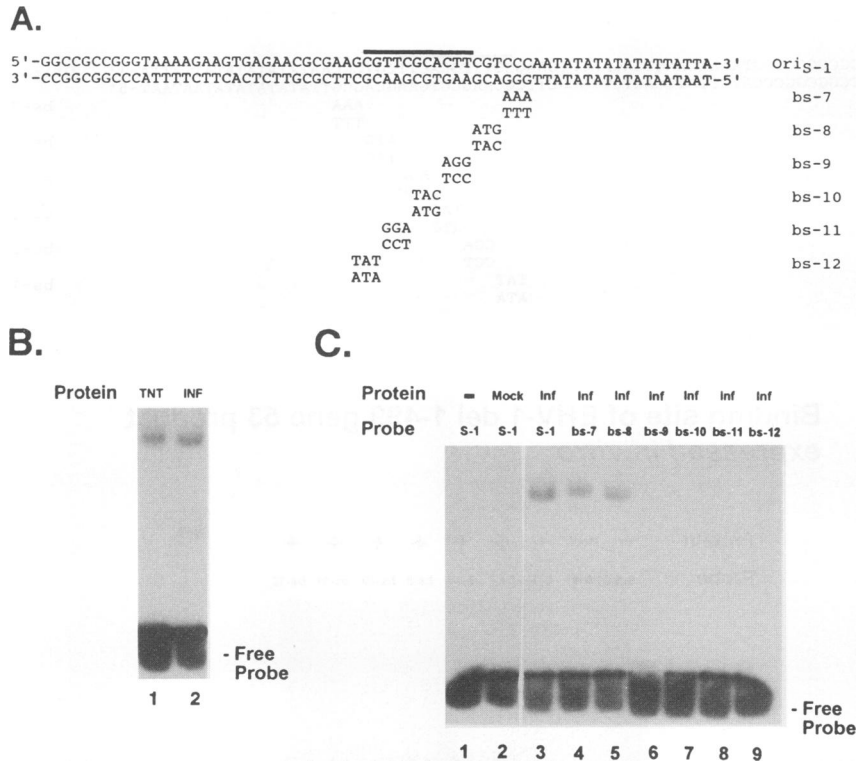

FIG. 9. Identification of an origin-binding activity from EHV-1-infected cells. (A) Sequence of HSV-1 Ori_{S-1} and triple base substitution mutants. The nucleotide sequences are shown along with the nature of base substitution mutants. (B) Binding of in vitro-expressed gene 53 product and infected nuclear extract (INF) proteins. Note similar rates of migration of the respective complexes formed with Ori_{S-1}. (C) Binding site determination for the infected nuclear extract protein. Proteins from infected cell extracts were used in gel shift assays with Ori_{S-1} (S-1) and its mutants as described in Materials and Methods. Note the similar retarded bands formed by the infected cell protein and the in vitro-expressed EHV-1 gene 53 product (lanes 3 to 9) as well as the lack of complex formation in the mock-infected extract (lane 2).

same helicase motif in all three proteins (Fig. 2). This zipper has the same arrangement of leucines in both EHV-1 and HSV-1. However, in the VZV OBP, the C-terminal leucine is replaced by alanine. The leucine zipper region was shown previously to be necessary for mediating higher order OBP-Ori_S complex formation (7, 12, 14b). In addition, HSV-1 OBP exists in solution as a dimer (3, 7, 9), and deletion of the amino-terminal half of the protein generates a protein that is a monomer in solution (18). These observations predict that the EHV-1 OBP leucine zipper may be important for higher-order interactions with the origin and that the protein will behave as a dimer in solution.

The carboxy-terminal half of the EHV-1 OBP also has regions of striking similarity to the HSV-1 and VZV OBPs. These regions are required for sequence-specific DNA binding (5, 22). Interestingly, EHV-1 OBP del 1–499 bound to HSV-1 Ori_{S-1} and recognized the same sequence as did the wild-type protein (Fig. 8). In addition, there were several regions that showed a high degree of amino acid similarity to the HSV-1 and VZV OBPs (Fig. 2). Results of mutagenesis of the HSV-1 OBP indicate that these regions are required for DNA binding (14a, 16). Thus, the functional conservation as well as the sequence conservation indicates that EHV-1, HSV-1, and VZV OBPs recognize similar DNA sequences via similar protein elements.

Taken together, our data suggest that the product of EHV-1 gene 53 is a homolog to the HSV-1 and VZV OBPs and possibly functions as the initiator of EHV-1 DNA replication. Perhaps, the proteins that initiate DNA replication in the alphaherpesviruses constitute a conserved family of proteins. Within this family, the amino-terminal half of the protein is responsible for the ATPase and helicase activities, as well as oligomerization via the leucine zipper. The C-terminal halves of the proteins are responsible for mediating sequence-specific binding to the origins of DNA replication. These findings open the way for a number of significant and very interesting experiments. Creation of chimeric OBPs will ultimately allow for the dissection of virus-specific and virus-general elements within both the origins of DNA replication and the OBPs required for initiation of DNA replication and will allow for construction of a basic model for initiation of alphaherpesvirus DNA replication.

ACKNOWLEDGMENTS

This work was supported by grants from the United States Department of Agriculture (No. 91-37204-6820) and the Elsa U. Pardee Foundation and by a research grant from the March of Dimes to Sumitra Deb. This work was done by Sumitra Deb during the tenure of an Established Investigatorship of the American Heart Association.

We thank Dennis O'Callaghan for the EHV-1 virus preparation as well as the EHV-1 origin-containing plasmid. We also thank Debbie Yrle for excellent typing assistance.

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