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Expression of the Varicella-Zoster Virus Origin-Binding Protein and Analysis of Its Site-Specific DNA-Binding Properties

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The varicella-zoster virus (VZV) genome contains homologs to each of the seven herpes simplex virus (HSV) genes that are required for viral DNA synthesis. VZV gene *51* is homologous to HSV *UL9*, which encodes an origin of DNA replication binding protein (OBP). It was previously shown, by using a protein A fusion protein, that the product of gene *51* is a site-specific DNA-binding protein which binds to sequences within the VZV origin (Stow et al., Virology 177:570–577, 1990). In this report, gene *51* was expressed in an in vitro translation system. Rabbit antiserum raised against the carboxyl-terminal 20 amino acids was used to confirm expression of the full-length gene *51* protein, and site-specific DNA-binding activity was demonstrated in a gel retardation assay. The origin-binding domain was located within a 263-amino-acid region of the carboxyl terminus by using a series of deletion mutants. The affinity of binding of the VZV OBP to the three binding sites in the VZV origin was found to be similar. In addition, as with UL9, a CGC triplet within a 10-bp consensus sequence is critical to the interaction between the OBP and the origin. The HSV and VZV OBPs, therefore, appear to have virtually identical recognition sequences despite only 33% identity and 44% similarity in the primary structure of their site-specific DNA-binding domains.

Mammalian DNA viruses have proven to be useful model systems for the study of eukaryotic DNA replication (3, 20). Herpesviruses, which have large linear DNA genomes, offer many advantages for DNA replication studies. Herpes simplex virus (HSV) is the best characterized of the herpesviruses. Its genome encodes about 72 genes, of which 7 have been shown to be required for viral DNA synthesis (2, 15, 18, 26). In addition, three origins of DNA replication have been identified, one copy of ori_L and two copies of ori_S (19, 21, 24, 25). Varicella-zoster virus (VZV) is a human herpesvirus which, like HSV, belongs to the subfamily *Alphaherpesvirinae* (14). Despite significant differences in their biologic behavior, VZV and HSV share many structural and molecular biological features. Sequence analysis has revealed that the 125-kb linear double-stranded DNA genome of VZV has homologs to all seven of the essential HSV DNA replication genes (5, 15). The VZV genome has no equivalent to HSV ori_L, but, like HSV, the VZV genome has two copies of ori_S in the inverted repeats flanking the short unique region of the genome (Fig. 1A).

VZV gene *51* is homologous to HSV *UL9* which encodes a protein that binds to the viral DNA origins of replication and is thought to play an important role in initiation of viral DNA synthesis (9, 17). The predicted amino acid sequence of the gene *51* protein was shown to be 44% identical to UL9, and it was expected that the product of gene *51* would be a VZV origin-binding protein (OBP) (5, 15). Consistent with this idea, a protein A-gene *51* fusion protein was shown to bind to the VZV origin, and, as with UL9, the origin-binding domain of the VZV OBP mapped to the carboxyl terminus (23). It is likely that the VZV OBP plays the same role as UL9 during viral DNA replication; however, there are no genetic data to support this idea and biochemical studies of the VZV OBP have been limited to the fusion protein mentioned above. An origin-binding activity has not been identified in extracts of VZV-infected cells, and expression of the full-length VZV OBP in a heterologous system has not been reported.

Despite both structural and functional similarities, the HSV and VZV origins differ in the arrangement of their OBP binding sites (Fig. 1B and C). HSV ori_S has two high-affinity binding sites which are part of an imperfect palindrome with a central AT-rich region. VZV ori_S also contains an AT-rich region, but it has three OBP binding sites which are all located on one side of the AT-rich region (4, 22, 23) (Fig. 1C). The significance of this structural difference between the VZV and HSV origins remains to be determined, but it is likely that comparative studies of the HSV and VZV DNA replication systems will contribute to our knowledge of the mechanisms involved in herpesvirus DNA synthesis. Specifically, studies on the VZV OBP not only will define its role in the initiation of VZV DNA replication, but will advance our understanding of the role of UL9 in HSV DNA replication. This approach is initiated in this report, in which the expression and functional characterization of the VZV OBP are described.

MATERIALS AND METHODS

Cloning of gene *51* into a vector for in vitro transcription and translation. The entire gene *51* open reading frame was cloned into pTM1, a plasmid originally constructed for making recombinant vaccinia viruses (16). pTM1 contains, in a 5′-3′ orientation, the bacteriophage T7 promoter, the encephalomyocarditis virus internal ribosome entry site which facilitates cap-independent ribosome binding, a multiple cloning site, translation stop codons in all three reading frames, and the T7 transcription terminal signal. A unique *Nco*I site, which contains an ATG, is 5′ of all other restriction enzyme sites within the multiple cloning site. Prior to cloning gene *51* into pTM1, the *Nco*I site and ATG were removed by digestion with *Nco*I, digestion of the 5′ protruding end with mung bean nuclease, and religation of the blunt ends to generate pMH4. The VZV

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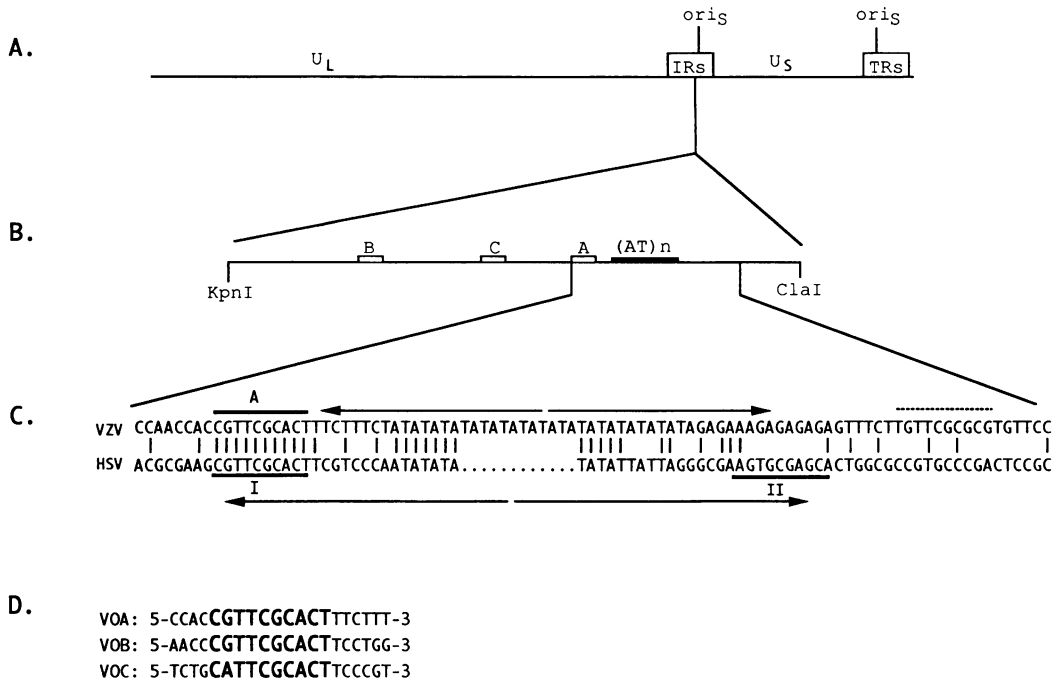


FIG. 1. Schematic of the VZV origin. (A) Location of the two copies of ori_S in VZV genome. (B) Structure of ori_S. Boxes labeled A, B, and C indicate the three *51* gene product binding sites which were determined by Stow et al. (23). (AT)n represents the AT-rich region. (C) Comparison of VZV and HSV origin sequences. The 46-bp palindromic sequence in the VZV origin is marked by divergent arrows above the top line. Note that the palindrome does not include site A which is indicated by a heavy line above the VZV sequence. HSV site I and site II are marked by a heavy line, and the palindrome, which includes both site I and site II, is marked by divergent arrows below the HSV sequence. The central dots in the HSV sequence indicate the shorter AT-rich region. The dashed line above the VZV sequence to the right of the AT-rich region shows a potential *51* binding site. (D) VOA, VOB, and VOC are three 20-mer oligonucleotides which contain the three binding sites A, B, and C, respectively, and which were used in the competitive binding assays in Fig. 8. The 10 bases in bold indicate the likely OBP recognition sequence.

gene *51* was obtained from a VZV genomic DNA library kindly provided by L. Gelb (Washington University). Prior to cloning gene *51* into the pMH4 vector, a *Bam*HI site was created just upstream of the start of the gene *51* open reading frame by using PCR-mediated mutagenesis. The entire gene *51*, from this *Bam*HI site (nucleotide sequence no. 87888) to a *Sty*I site (nucleotide sequence no. 90513), was cloned, using *Bam*HI linkers, into the *Bam*HI site of pMH4 to generate pMH4-51.

Construction of deletion mutants of the gene *51*. Nine deletion mutants of gene *51* were constructed in pTM1. Figure 2 shows schematically the position of each deletion. Prior to making the deletion mutations, an *Nco*I site was created at the 5′ end of the gene *51* open reading frame by using PCR-mediated mutagenesis. Gene *51* was then cloned into the *Nco*I and *Bam*HI sites of pTM1 to generate pTM151. Five gene *51* amino-terminal deletion mutants were generated from pTM151 by digestion with *Nco*I and five restriction enzymes with sites within the gene *51* open reading frame (*Aat*II, *Nde*I, *Pvu*II, *Dra*I, and *Spe*I). An additional deletion mutant was generated by using an *Nco*I site created by PCR-mediated mutagenesis. Three carboxyl-terminal deletion mutants were generated starting with pTM151-*Pvu*II, one of the amino-terminal mutants, by digestion with one of three restriction sites within the gene *51* open reading frame (*Bgl*II, *Eco*47III, and *Sma*I) and a *Stu*I site at the 3′ end of the multiple cloning site in the vector, followed by religation. The positions of these restriction sites within the gene *51* open reading frame are shown in Fig. 2.

Production of antisera against the C terminus of the gene *51* product. A 20-amino-acid peptide, CELSPGSWPRINGAVN FESL, whose sequence was derived from the predicted carboxyl terminus of the gene *51* open reading frame, was synthesized by the Protein Chemistry Laboratory at Washington University. This peptide was conjugated to keyhole limpet hemocyanin by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Corp., Rockford, Ill.). The conjugated peptide was used to immunize rabbits (1 mg per rabbit in three injections). The activity of the antiserum was tested by immunoblot analysis using gene *51* protein made in insect cells infected with a recombinant baculovirus. One such rabbit antiserum (R309) and the preimmunization serum from the same rabbit (pre-R309) were used for all immunoprecipitation and "double-retardation" experiments described.

In vitro transcription and translation of gene *51*. All in vitro transcription-translation reactions were done by using the TnT coupled reticulocyte lysate system and T7 RNA polymerase according to the manufacturer's recommendations (Promega Corp., Madison, Wis.). Four microliters of L-[^{35}S]methionine (1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added to the 50-μl reaction to generate radiolabeled in vitro synthesized proteins. The same protocol was used to synthesize unlabeled gene *51* protein for use in gel retardation assays except that additional L-methionine was used by adding 1 μl of the leucine-minus amino acid mix provided by the supplier in place of L-[^{35}S]-methinone.

SDS-PAGE and autoradiography. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of L-[^{35}S]

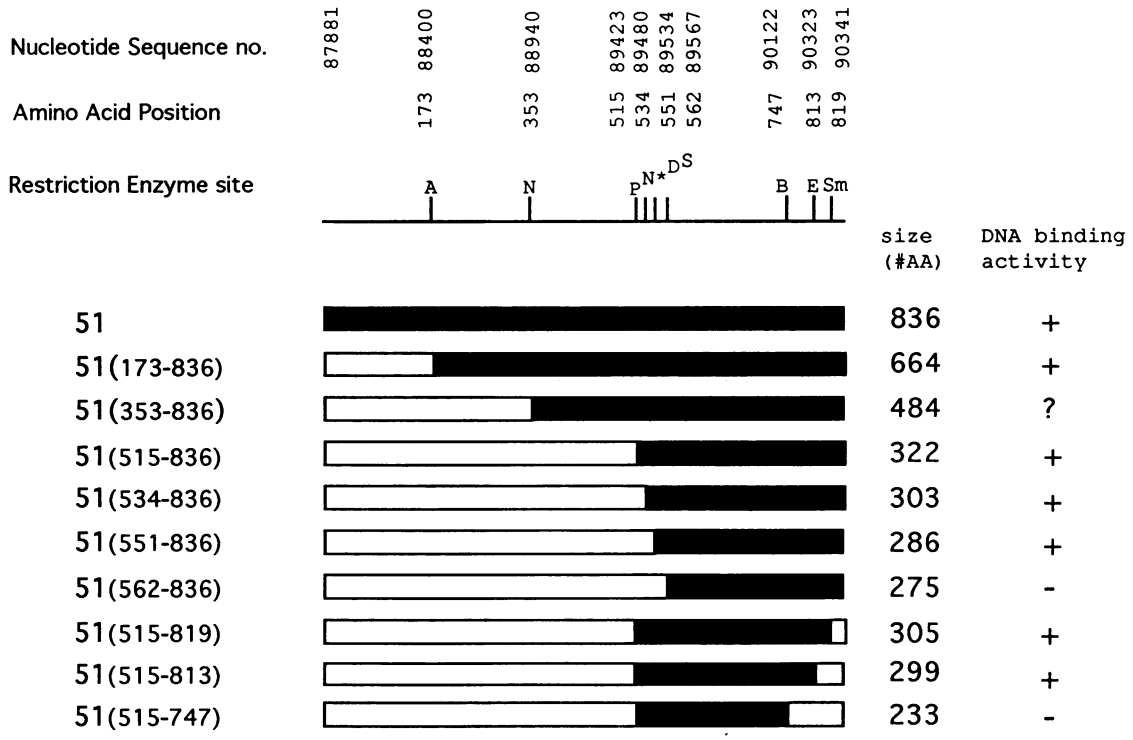


FIG. 2. Schematic representation of VZV gene *51* deletion mutants. White bars indicate the regions deleted. Nucleotide sequence no. refers to the genomic sequence of Davison and Scott (5). Amino acid position, in the case of the amino-terminal deletions, indicates the residue which immediately follows the initiation methionine derived from the vector. In the case of the carboxyl-terminal deletions, amino acid position indicates the last residue of the mutant polypeptide derived from the *51* sequence. Restriction sites used to generate the mutants are shown by letters. A, *Aat*II; N, *Nde*I; P, *Pvu*II; S, *Spe*I; D, *Dra*I; B, *Bgl*II; E, *Eco*47III; Sm, *Sma*I. N* represents an *Nco*I site which is not present in the wild-type sequence of gene *51* but which was generated by PCR mutagenesis to derive the $51_{(534-836)}$ deletion mutant. The sizes represent the number of amino acids in the mutant translation products. The DNA-binding activity was determined by gel retardation assays as shown in FIG. 3 and FIG. 6. +, binding activity; −, no binding activity; ?, ambiguous result.

methionine-labeled in vitro-synthesized proteins was carried out as described in Promega's Protocols And Applications Guide. After electrophoresis, the gel was fixed, soaked in Amplify solution (Amersham Corp.), dried, and exposed to X-ray film (Fuji RX film; Fisher Scientific, St. Louis, Mo.) at −70°C.

Immunoprecipitation. One microliter of antiserum R309 or preimmune antiserum (pre-R309) was coupled to 20 μl of protein A-Sepharose (Sigma Co., St. Louis, Mo.) in 300 μl of phosphate-buffered saline with 0.1% Tween 20 (PT buffer) in a rotator overnight at 4°C. The coupled Sepharose beads were washed three times with PT buffer and once with PT buffer containing 5 mg of bovine serum albumin per ml (PTB buffer). The L-[^{35}S]methionine-labeled in vitro translation products were added to a tube containing the antiserum-coupled Sepharose in 300 μl of PTB buffer. After being rotated for 2 h at 4°C, the Sepharose beads were washed three times with PTB buffer and once with PT buffer. SDS-PAGE loading buffer was added to the pellet, and after being boiled for 2 min, the Sepharose beads were centrifuged and the supernatant was analyzed by SDS-PAGE (10% acrylamide) and autoradiography.

Oligonucleotide probes used in the gel retardation assays. A 26-base oligonucleotide containing site A of the VZV replication origin (VOA-26f) (5′-CCAACCACCGTTCGCACTTTC TTTCT-3′) and a complementary oligonucleotide (VOA26r) were synthesized by the Protein Chemistry Laboratory of Washington University. VOA-26f was radiolabeled at the 5′ end with [γ-^{32}P]ATP (4,500 Ci/mmol; Amersham Corp.) and T4 polynucleotide kinase (Promega Corp.). A double-stranded site A oligonucleotide (VOA-26) was prepared by incubating radiolabeled VOA-26f with excess unlabeled VOA-26r in annealing buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$), for 10 min at 65°C, 10 min at 55°C, 15 min at 42°C, and 60 min at 37°C. Unlabeled 20-mer double-stranded oligonucleotides used in the competition gel retardation assays were annealed in an identical manner.

Gel retardation assays. The DNA-protein binding reaction was performed at room temperature for 15 min. Each reaction was done with 2 to 4 μl of in vitro-synthesized protein, 10^4 cpm of radiolabeled probe (0.8×10^6 to 1.0×10^6 cpm/μg) and 1 μg of poly(dI-dC) (Pharmacia Corp., Piscataway, N.J.) in a final volume of 25 μl. The incubation buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], [pH 7.9], 5 mM KCl, 2.5 mM $MgCl_2$, 33.3 mM NaCl, 20 mM Tris [pH 7.8], 3.8 mM EDTA, 5% glycerol, 0.03% Tween 20, 1.2 mM 2-mercaptoethanol) and electrophoresis buffer (28.54 g of glycine, 6.04 g of Tris base, 4 ml of 0.5 M EDTA per liter) were made according to a commercial protocol (Stratagene, La Jolla, Calif.). After incubation, samples were loaded onto a 6% native polyacrylamide gel (acrylamide:bisacrylamide ratio of 80:1). After electrophoresis at 4°C for 1 h with a constant current of 20 mA, the gel was dried and exposed to X-ray film. For double-retardation experiments, 0.2 to 0.5 μl of antiserum was added at the start of the reaction.

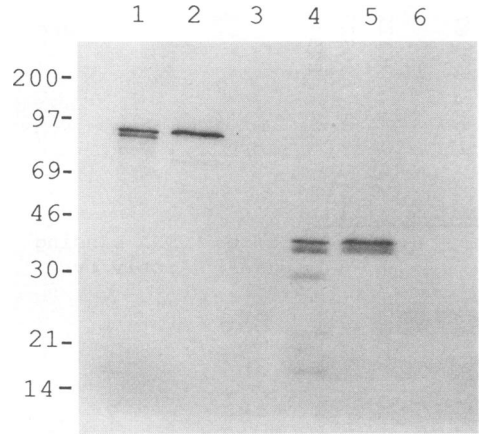


FIG. 3. Immunoprecipitation and SDS-polyacrylamide gel analysis of L-[^{35}S]methionine-labeled *51* gene product synthesized in vitro by using a reticulocyte lysate. Lane 1, full-length product of VZV gene *51*; lane 2, immunoprecipitation of full-length product of VZV gene *51* by anti-51 antiserum R309; lane 3, immunoprecipitation of full-length product of VZV gene *51* using preimmune antiserum (pre-R309); lane 4, mutant $51_{(515-836)}$ product (Fig. 2); lane 5, immunoprecipitation of $51_{(515-836)}$ product by R309 antiserum; lane 6, immunoprecipitation of $51_{(515-836)}$ product by preimmune antiserum (pre-R309). The sizes (kilodaltons) and positions of molecular weight markers are indicated on the left.

Competitive gel retardation assays. Competitive gel retardation experiments were performed with the same conditions as standard gel retardation assays except that unlabeled competitor oligonucleotides were added at the start of the binding reaction. The dried gels were submitted to a BetaScope 603 Blot Analyzer (Betagen, A Division of Intelligenetics, Inc., Mountain View, Calif.) to quantify the radioactivity in the protein-DNA complex. The analysis was performed according to the manufacturer's instructions, with a recording time of 40 min for each gel.

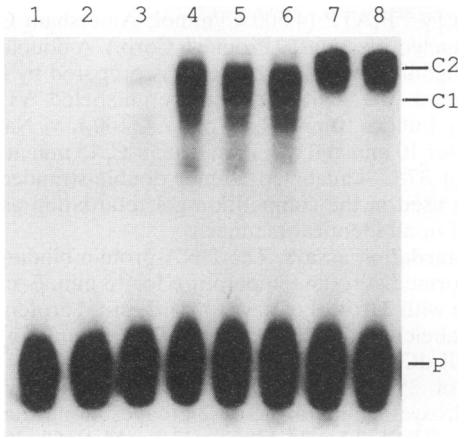


FIG. 4. Gel retardation assay demonstrating the site-specific binding activity of in vitro translated product of VZV gene *51*. The probe is a 26-mer double-stranded oligonucleotide (VOA-26) which corresponds to the DNA sequence at site A (see Materials and Methods). Lane 1, control lysate; lanes 2 and 3, control lysate with 0.2 and 0.5 μl of anti-51 antiserum R309, respectively; lane 4, control lysate with gene *51* product; lanes 5 and 6, gene *51* product with 0.2 and 0.5 μl of control antiserum (pre-R309), respectively; lanes 7 and 8, *51* gene product with 0.2 and 0.5 μl of anti-51 antiserum (R309), respectively. P, free probe; C1, DNA probe-protein complex; C2, DNA probe-protein-antibody complex.

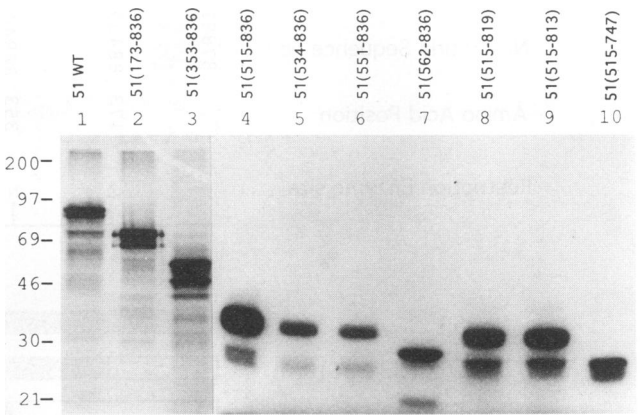
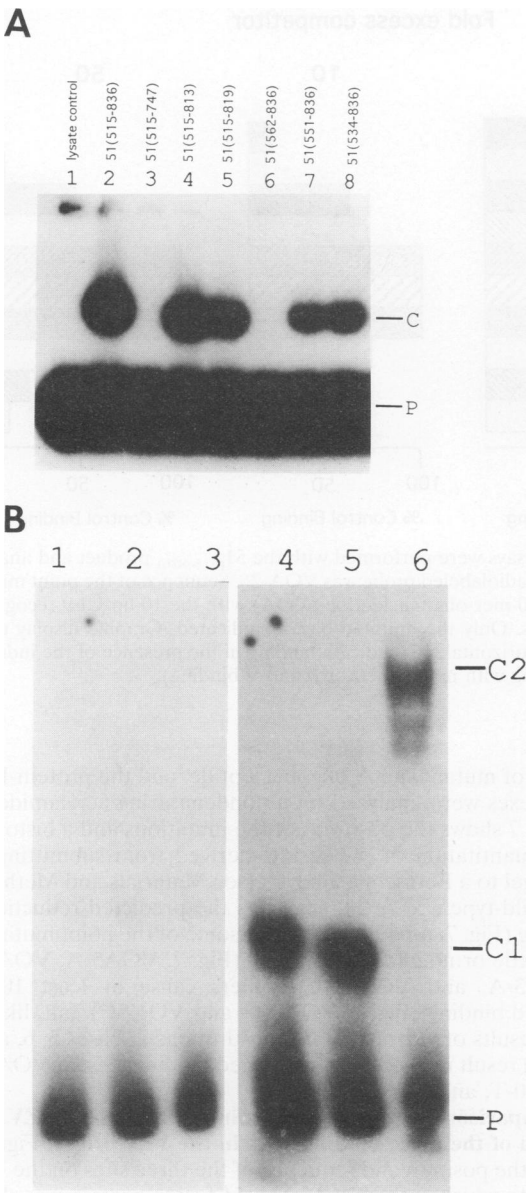


FIG. 5. SDS-polyacrylamide gel analysis of L-[^{35}S]methionine-labeled products of gene *51* deletion mutants synthesized in vitro. Lysate (2 μl) was loaded into each lane. Lane 1, full-length gene 51 product; lane 2, $51_{(173-836)}$ product; lane 3, $51_{(353-836)}$ product; lane 4, $51_{(515-836)}$ product; lane 5, $51_{(534-836)}$; lane 6, $51_{(551-836)}$; lane 7, $51_{(562-836)}$; lane 8, $51_{(515-819)}$; lane 9, $51_{(515-813)}$; lane 10, $51_{(515-747)}$. The sizes (kilodaltons) and positions of the molecular weight markers are indicated on the left.

RESULTS

In vitro transcription and translation of VZV gene *51*. A 2.7-kb fragment from the VZV genome containing the entire gene *51* coding sequence was subcloned into a vector suitable for in vitro transcription and translation as described in Materials and Methods section. The L-[^{35}S]methionine-labeled translation product of gene *51* was analyzed by SDS-PAGE and immunoprecipitation followed by SDS-PAGE. The most slowly migrating protein band (Fig. 3, lane 1) corresponds to a molecular weight of 85,000. This in vitro synthesized protein comigrated with gene *51* protein expressed in insect cells infected with a recombinant baculovirus (data not shown). The predicted molecular size of gene *51* protein is 94,314, however, and the possibility that the 85-kDa polypeptide was derived from proteolytic degradation or internal translation initiation was considered. The ^{35}S-labeled translation product was then immunoprecipitated with a rabbit antiserum (R309) which recognizes the carboxyl-terminal 20 amino acids of gene *51* protein. The 85-kDa protein was immunoprecipitated by R309 but not by a preimmune antiserum from the same rabbit (Fig. 3, lanes 2 and 3). R309 antiserum also immunoprecipitated a 322-amino-acid polypeptide from the carboxyl terminus [$51_{(515-836)}$; Fig. 2 and Fig. 3, lane 5] but not a peptide with a deleted carboxyl terminus [$51_{(515-819)}$); Fig. 2 and data not shown], showing the specificity of the R309 antisera for the carboxyl terminus. Amino-terminal amino acid sequence analysis was performed on the 85-kDa gene *51* protein expressed in recombinant baculovirus-infected insect cells. Nine of ten residues from the amino terminus were determined unambiguously and were identical to the predicted amino acid sequence encoded by gene *51* (5). The 85-kDa protein therefore represents the full-length product of gene *51*.

DNA-binding activity of in vitro-synthesized gene *51* protein. The 85-kDa gene *51* product obtained from extracts of insect cells infected with a recombinant baculovirus was insol-



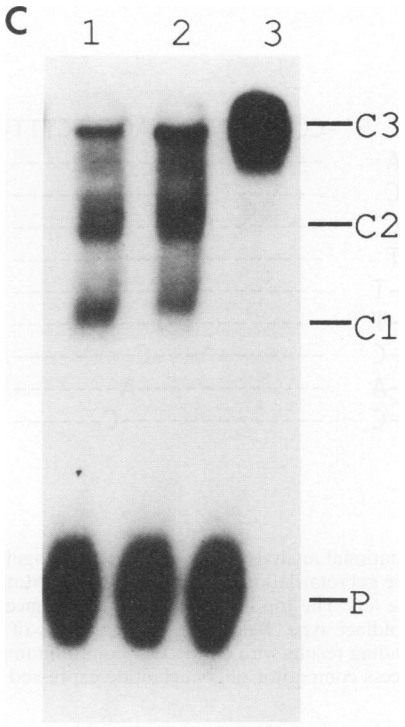


FIG. 6. Gel retardation assays using in vitro synthesized products of gene *51* deletion mutants. The probe (VOA-26) was the same as used in Fig. 3. (A) Lane 1, control lysate; lane 2, $51_{(515-836)}$ product; lane 3, $51_{(515-747)}$ product; lane 4, $51_{(515-813)}$ product; lane 5, $51_{(515-819)}$ product; lane 6, $51_{(562-836)}$ product; lane 7, $51_{(551-836)}$ product; lane 8, $51_{(534-836)}$ product. P, free probe; C, probe-protein complex. (B) Double retardation using $51_{(515-836)}$ mutant product. Lane 1, control lysate; lane 2, control lysate with 0.2 μl of pre-R309; lane 3, control lysate with 0.2 μl of R309; lane 4, lysate with $51_{(515-836)}$ product; lane 5, $51_{(515-836)}$ product with 0.2 μl of pre-R309; lane 6, $51_{(515-836)}$ product with 0.2 μl of R309. P, free probe; C1, probe-protein complex; C2, protein-antibody probe complex. (C) Double retardation with $51_{(173-836)}$ mutant product. Lane 1, $51_{(173-836)}$ product; lane 2, $51_{(173-836)}$ product with 0.2 μl of control serum (pre-R309); lane 3, $51_{(173-836)}$ product with 0.2 μl of anti-51 antiserum (R309). P, free probe; C1 and C2, protein-probe complexes; C3, protein-antibody-probe complex.

uble and therefore not suitable for functional studies. The gene *51* protein synthesized in vitro was tested, therefore, for site-specific DNA-binding activity in a gel retardation assay. A 5′ ^{32}P-end-labeled 26-mer probe (VOA-26) was used in the gel retardation assay (see Materials and Methods). Figure 4 shows that the rabbit reticulocyte extract containing in vitro-translated gene *51* protein (lane 4) caused a retardation of the probe whereas the control rabbit reticulocyte lysate displayed no retarded bands (lane 1). The retarded complex(es) did not yield a discrete band, but addition of anti-51 antisera (R309) led to a double retardation of all the complex(es) (lanes 7 and 8).

Determination of the boundaries of the site-specific DNA-binding domain of the VZV OBP. The site-specific DNA-binding domain of the gene *51* product was mapped to the carboxyl-terminal 322 amino acids with a protein A fusion protein (23). To confirm this in the in vitro translation system and to determine whether the DNA-binding domain could be expressed as a stable polypeptide, a series of deletion mutants of the gene *51* were constructed. Figure 2 shows a schematic map of these mutants and their relationship to the full-length OBP. Figure 5 shows an autoradiograph of the L-[^{35}S]methionine-labeled in vitro translation products of each deletion mutant analyzed by SDS-PAGE. Although multiple radiolabeled products were seen with each mutant, the most slowly migrating band corresponds to the predicted size of each mutant polypeptide.

To determine the boundary of the DNA-binding domain of the VZV OBP, each mutant product was tested for site-specific DNA-binding activity. Figure 6 shows an autoradiograph of the gel retardation results which are summarized in Fig. 2. The smaller polypeptides gave a single retarded complex (Fig. 6A, lanes 2, 4, 5, 7, and 8, and Fig. 6B, lanes 4 and 5). The larger polypeptides [e.g., $51_{(353-836)}$] did not yield a definite protein-DNA complex (data not shown; indicated by a question mark in Fig. 2) or yielded several indistinct retarded bands [$51_{(173-836)}$; Fig. 6C, lane 1]. To demonstrate that these indistinctly retarded complexes contained truncated gene *51*

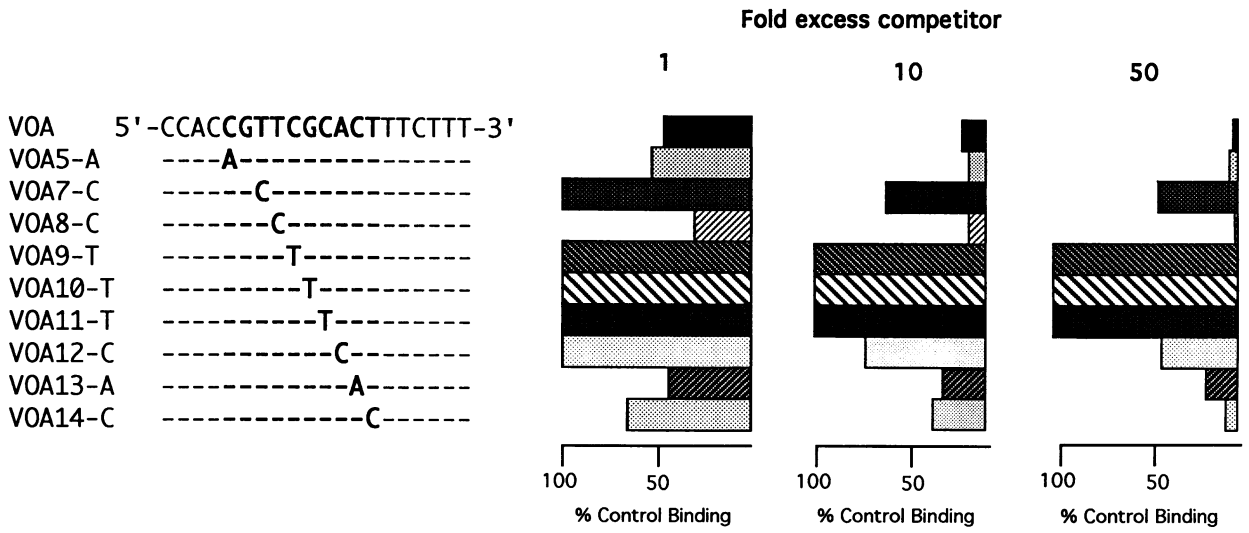


FIG. 7. Mutational analysis of the VZV OBP recognition sequence. Binding assays were performed with the $51_{(515-836)}$ product and analyzed by a competitive gel retardation assay as described in Materials and Methods. The radiolabeled probe was VOA-26. Sequence of the point mutants is shown on the left. The top line indicates the sequence of the wild-type site A 20-mer oligonucleotide (VOA) with the 10-bp UL9 recognition sequence in boldface type. Below are the sequences of a series of point mutants. Only the mutated base is indicated. Graphic display of the competitive binding results with each VOA point mutants is shown on the right. Horizontal bars indicate binding in the presence of the indicated amounts of excess competitor oligonucleotide expressed as a percentage of binding with no competitor (control binding).

polypeptides with an intact carboxyl terminus, a double-retardation assay was performed by using the anti-carboxyl-terminal antisera (R309). A double retardation assay with truncated polypeptides $51_{(515-836)}$ (Fig. 6B, lane 6) and $51_{(173-836)}$ (Fig. 6C, lane 3) showed that all the complexes appear to be double-retarded by R309 antiserum but not by preimmune antiserum (Fig. 6B, lane 5; Fig. 6C, lane 2). The amino terminus of the DNA-binding domain of gene *51* protein is located between amino acids 551 and 562, and the carboxyl terminus is located between amino acids 747 and 813 (Fig. 2). The smallest polypeptide tested which retained DNA-binding activity [$51_{(551-836)}$; Fig. 2] was 286 amino acids, but the DNA-binding domain may be as small as 263 amino acids.

Effect of single-base-pair mutations in the VZV OBP recognition sequence. Elias et al. identified an 11-bp sequence, 5′-CGTTCGCACTT-3′ that is required for high-affinity binding of UL9, and Hazuda et al. refined the UL9 recognition sequence to 10 bp (CGTTCGCACT) (7, 11). In the VZV origin this 10 bp sequence is found within site A and site B, and site C contains 9 of the 10 bp. Stow et al. showed that these bases are well protected in DNase I footprinting experiments using a fusion protein containing 322 amino acids from the carboxyl terminus of the OBP and a DNA fragment that contained site A and site C (23). In addition, deletion of these 10 bases eliminated binding. Hazuda et al. performed saturation mutagenesis on the 10-bp UL9 recognition sequence to assess the relative contribution of each base pair. These authors showed that any point mutation within the 10-bp sequence reduced binding by 2- to 10-fold, but that any substitution at positions 5, 6, and 7 (CGC) reduced UL9 binding by more than 100-fold (11).

In the present work, nine 20-mer oligonucleotides each containing a single point mutation in this 10-base sequence within the context of the site A sequence were made and tested for gene *51* protein binding in a competitive gel retardation assay. In vitro synthesized $51_{(515-836)}$ product was incubated with radiolabeled VOA-26 in the presence or absence of an excess of mutant site A oligonucleotide, and the protein-DNA complexes were analyzed on a nondenaturing acrylamide gel. Figure 7 shows the positions of the mutations and a histogram of a quantitation of the results derived from submitting the dried gel to a Betascope analysis (see Materials and Methods). The wild-type site A 20-mer gives the predicted reduction in binding (Fig. 7, top line). Whereas some of the point mutations have little or no effect on binding (Fig. 7, VOA5-A, VOA8-C, VOA13-A, and VOA14-C), others cause at least 10-fold reduced binding (Fig. 7, VOA7-C, and VO12-C), but, like the UL9 results of Hazuda et al., substitutions at bases 5, 6, and 7 (CGC) result in over 50-fold reduced binding (Fig. 7, VOA9-T, VOA10-T, and VOA11-T).

Comparison of the relative binding affinity of the VZV OBP to each of the three binding sites in the VZV origin. Figure 1 shows the position and sequence of the three sites on the VZV origin to which a protein A-gene *51* fusion protein was shown to bind (23). The relative binding affinity of the VZV OBP to these three sites was analyzed by using a competitive gel retardation assay. In vitro synthesized $51_{(515-836)}$ product was used for this analysis. Three 20-mer oligonucleotides, which correspond to site A (VOA), B (VOB), and C (VOC), were used to compete for binding with a radiolabeled 26-mer oligonucleotide corresponding to the DNA sequence of site A (VOA-26). Figure 1D shows the DNA sequence of these oligonucleotides. Figure 8A shows the autoradiographic results, and Fig. 8B shows a graphic display of a quantitative analysis of the results derived from submitting the dried gel to a BetaScope. The VZV OBP binds to sites A and B with virtually identical affinities, but it binds to site C with a two- to threefold lower affinity. This same result was obtained on three separate experiments and by labeling a site C oligonucleotide (VOC) and competing with VOA (data not shown). Sequence comparison of the three sites with respect to the 10-base recognition sequence shows that, whereas sites A and B are identical, the second base pair of the site C 10-mer recognition sequence is A instead of G (Fig. 1D). A 20-bp oligonucleotide

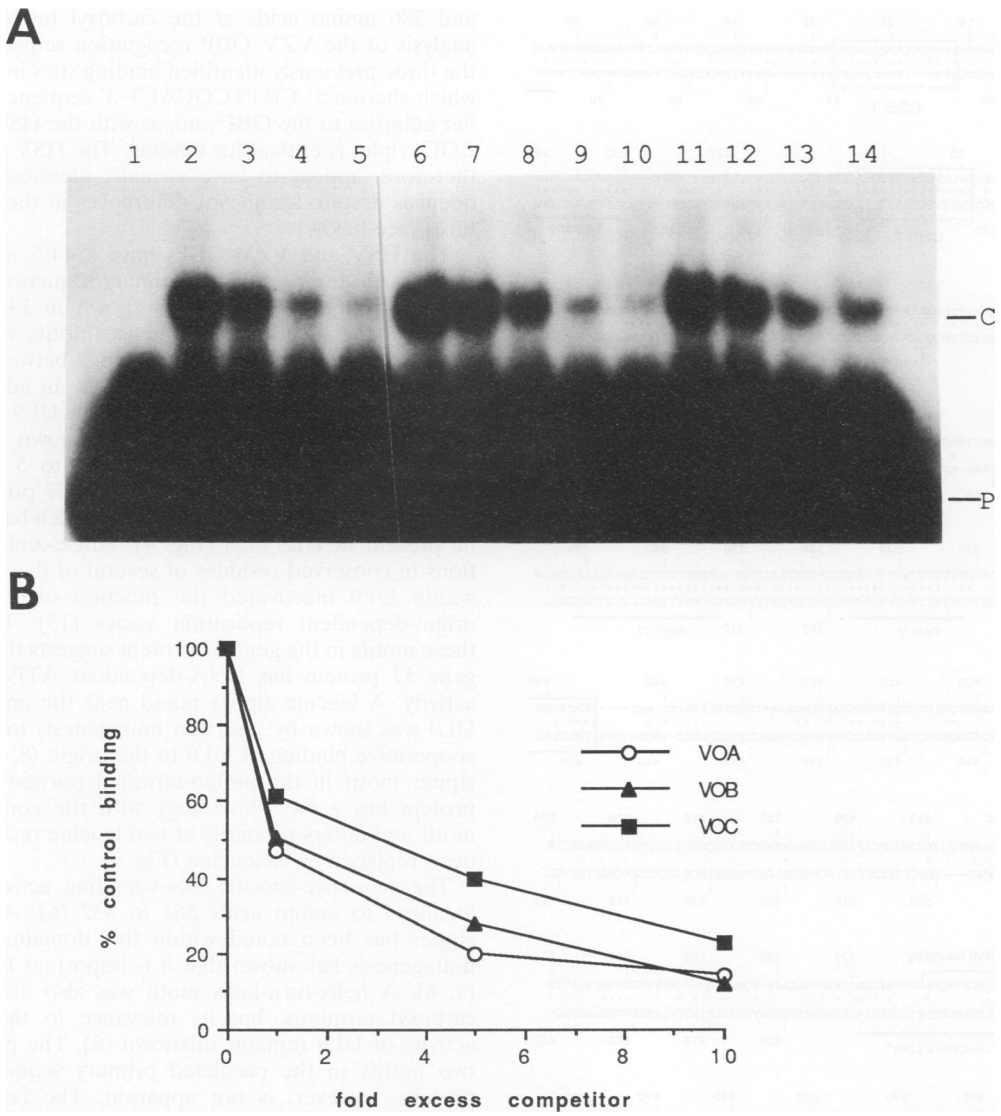


FIG. 8. Comparison of the binding affinity of the VZV OBP to the three binding sites in the VZV origin. Competitive gel retardation assays were performed as described in Materials and Methods. Binding assays were performed with the $51_{(515-836)}$ in vitro translation product. VOA, VOB, and VOC 20-mer probes were used as competitor oligonucleotides (Fig. 1D). (A) Autoradiograph. Lane 1, control lysate; lanes 2 and 6, $51_{(515-836)}$ product without competitor; lane 3, addition of an equimolar amount of unlabeled VOA; lane 4, fivefold excess VOA; lane 5, 10-fold excess VOA; lane 7, equimolar unlabeled VOB; lane 8, 5-fold excess VOB; lane 9, 10-fold excess VOB; lane 10, 20-fold excess VOB; lane 11, equimolar VOC; lane 12, 5-fold excess VOC; lane 13, 10-fold excess VOC; lane 14, 20-fold excess VOC. P, free probe; C, probe-protein complex. (B) Graphic display of the competitive gel retardation data derived from the results shown in panel A. The amount of binding in the presence of the indicated amounts of excess competitor oligonucleotide divided by the amount of binding with no competitor times 100 (% control binding) is plotted against the molar ratio of unlabeled competitor VOA, VOB, and VOC to radiolabeled probe (fold excess competitor).

corresponding to site C except for a single A to G mutation was made, and by using a competitive binding assay, it was found that the binding affinity was identical to site A and B (data not shown). This result is consistent with the idea that the small difference in binding affinity to site C is due to the G to A difference in the 10-bp recognition sequence rather than differences in the flanking sequences.

DISCUSSION

In this report, the expression and functional identification of the product of VZV gene *51* are described. The full-length gene *51* protein, expressed in a rabbit reticulocyte lysate in vitro transcription-translation system, has a molecular weight of 85,000 as determined by SDS-PAGE, despite a predicted molecular weight of 94,314. This is similar to UL9, which also exhibits an unexplained, faster-than-predicted mobility in SDS-PAGE. Gel retardation results indicated that the full-length gene *51* protein binds to specific sequences found within the VZV origin of replication. However, like UL9, full-length gene *51* protein does not yield a discrete protein-DNA complex in native polyacrylamide gels whereas carboxyl-terminal polypeptides of both OBPs do yield discrete bands in this assay. In vitro translation products of a series of deletion mutants were analyzed for site-specific DNA-binding activity, and the results were used to localize the origin-binding domain to between 263

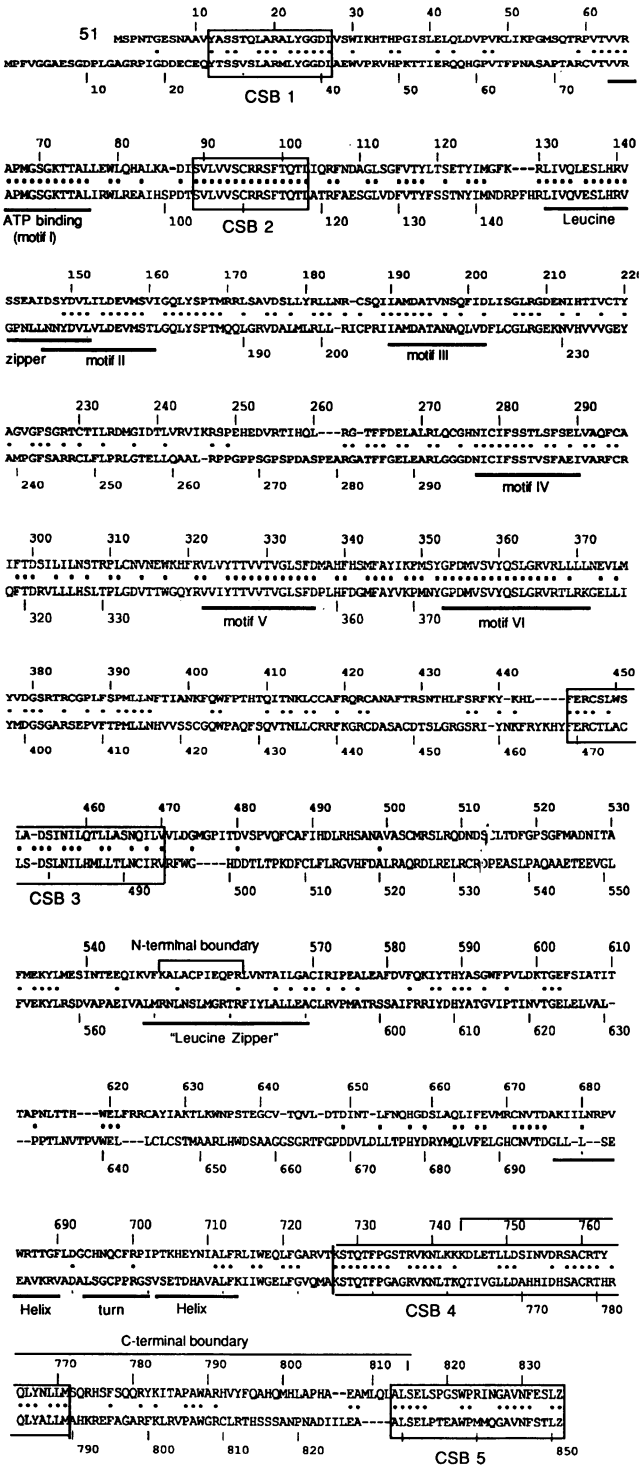


FIG. 9. Alignment of the predicted amino acid sequences of the VZV gene *51* protein and HSV UL9. The top line shows the amino acid sequence of the gene *51* protein, and the bottom line shows the UL9 sequence. Bars below the UL9 sequence indicate the positions of sequence motifs previously noted in UL9 (6, 13). Motifs I, II, III, IV, V, and VI represent helicase superfamily motifs. The N-terminal and C-terminal boundaries indicated above the gene *51* protein sequence represent the boundaries of the site-specific DNA-binding domain. The outermost point of each boundary retained DNA-binding activity, and the innermost point lacked DNA binding. The boxes marked CSB 1 through CSB 5 indicate conserved sequence blocks which are arbitrarily marked regions of high (≥60%) homology.

and 286 amino acids at the carboxyl terminus. Finally, an analysis of the VZV OBP recognition sequence showed that the three previously identified binding sites in the VZV origin, which share a 5′-CRTTCGCACT-3′ sequence, bind with similar affinities to the OBP and, as with the HSV OBP (UL9), a CGC triplet is critical for binding. The HSV and VZV OBPs, therefore, appear to have virtually identical recognition sequences despite significant differences in their primary structures (see below).

The HSV and VZV OBPs have a 44% identity and 53% similarity in their predicted primary sequences. An alignment of their primary sequences is shown in Fig. 9, which also indicates the location of sequence motifs which have been noted in UL9. Most of the homology between UL9 and the VZV OBP occurs within these motifs. In addition, there are five regions of high homology between UL9 and the gene *51* protein which do not occur within known sequence motifs (conserved sequence blocks [CSB] 1 to 5 in Fig. 9). The amino-terminal two-thirds of the gene *51* protein contains all six of the helicase superfamily motifs which have been noted to be present in UL9 (13) (Fig. 9). Single-amino-acid substitutions in conserved residues of several of these helicase motifs within UL9 inactivated the function of UL9 in transient origin-dependent replication assays (13). The presence of these motifs in the gene *51* protein suggests that, like UL9, the gene *51* protein has DNA-dependent ATPase and helicase activity. A leucine zipper noted near the amino terminus of UL9 was shown by insertion mutagenesis to be required for cooperative binding of UL9 to the origin (8, 11). The leucine zipper motif in the amino-terminal portion of the gene *51* protein has a 64% homology with the corresponding UL9 motif and differs primarily at two leucine residues which have been replaced by isoleucine (Fig. 9).

The sequence-specific DNA-binding activity of UL9 was localized to amino acids 564 to 832 (6). A pseudo-leucine zipper has been noted within this domain, and insertional mutagenesis has shown that it is important for DNA binding (1, 6). A helix-turn-helix motif was also identified near the carboxyl terminus, but its relevance to the DNA binding activity of UL9 remains unknown (6). The presence of these two motifs in the predicted primary sequence of gene *51* protein, however, is not apparent. The boundaries of the origin-binding domain of the gene *51* protein are very similar to those determined for UL9, but the homology of the origin-binding domains is actually less (33% identity and 44% similarity) than the overall homology of the two molecules. The sequence requirements for binding of both OBPs, however, are virtually identical, and each OBP binds to both origins (23 and unpublished observation). There are two regions of high homology in the origin-binding domain. The region of homology referred to here as CSB 4 was recently shown to be important for, but not sufficient for, UL9 DNA binding (12). Whether these conserved amino acids are directly or indirectly involved in sequence recognition remains to be determined. CSB 5 at the carboxyl terminus is not required for DNA binding of either protein (Fig. 2) (1, 6). This suggests that the origin-binding domain is involved in an as-yet-undetermined function common to both OBPs.

HSV ori_S and VZV ori_S have very similar OBP recognition sequences, but an apparent difference in the arrangement of the binding sites relative to their AT-rich regions (Fig. 1).

Whereas HSV ori_S has a high-affinity binding site on each side of the AT-rich region, the VZV origin contains three origin-binding sites which are all on the same side of the AT-rich region. The VZV OBP was shown to bind with equal affinity to site A and site B and with a slightly lower affinity to site C. The similar binding affinity of all three sites is interesting given that plasmid replication assays indicated that site A alone is sufficient for plasmid replication in VZV-infected cells (23). This structural difference between the HSV and VZV origins is also interesting in light of the observation that plasmids containing the VZV origin are replicated in HSV-infected cells (22). This suggests that the apparent difference in arrangement of the binding sites is not critical to the formation of a functional initiation complex. Alternatively, it is possible that there are as-yet-undetected binding sites to the right of the AT-rich region in VZV ori_S. By using an oligonucleotide probe derived from sequences to the right of the AT-rich region, which contain 7 of the 10 bp of the recognition sequence (see dashed line, Fig. 1C), no binding was observed (data not shown). A very low affinity binding site, however, could be missed in binding assays using DNA probes which contain a single binding site or in DNase I footprint assays using gene *51* protein lacking the amino terminus (23). The amino terminus of UL9 has been shown to be required for cooperative binding, and binding to a low-affinity site (site III) has been observed only in DNase I footprints of full-length UL9 on DNA fragments which also contain site I (7, 8, 10). Identification of low-affinity gene *51* protein binding sites on VZV ori_S may, therefore, depend upon binding studies with full-length gene *51* protein.

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