JOURNAL OF VIROLOGY, July 1994, p. 4126–4136
0022-538X/94/$04.00+0

Vol. 68, No. 7

Alphaherpesvirus Origin-Binding Protein Homolog Encoded by Human Herpesvirus 6B, a Betaherpesvirus, Binds to Nucleotide Sequences That Are Similar to *ori* Regions of Alphaherpesviruses

NAOKI INOUE,[1]† TIMOTHY R. DAMBAUGH,[2]‡ JEFFREY C. RAPP,[1] AND PHILIP E. PELLETT[1]*

Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333,[1] and Dupont Merck Pharmaceuticals Co., Wilmington, Delaware 19880[2]

Received 23 November 1993/Accepted 21 March 1994

We previously identified a human herpesvirus 6B (HHV-6B) homolog of the alphaherpesvirus origin-binding protein (OBP), exemplified by the herpes simplex virus type 1 UL9 gene product. This finding is of particular interest because HHV-6B is otherwise more closely related to members of the betaherpesvirus subfamily. The prototypic betaherpesvirus, human cytomegalovirus, does not encode an obvious OBP homolog and contains a more complex origin of replication than do alphaherpesviruses. Thus, analysis of the function of the HHV-6B OBP homolog is essential for understanding the mechanism of HHV-6B DNA replication initiation. The HHV-6B OBP homolog, OBP_{H6B}, was expressed in vitro by coupled transcription and translation and in insect cells by infection with recombinant baculoviruses. The expressed protein bound to two DNA sequences located upstream of the HHV-6B major DNA-binding protein gene homolog, within a region that was predicted to serve as an origin of replication on the basis of its sequence properties. The binding sites lie within 23-bp segments and are similar to OBP-binding sites of herpes simplex virus type 1. The two OBP_{H6B}-binding sequences are separated by an AT-rich region and have an imperfect dyad symmetry as do the alphaherpesvirus origin regions. We identified OBP_{H6B} transcripts by reverse transcription PCR in HHV-6B-infected Molt-3 cells. These results suggest that OBP_{H6B} functions in a manner analogous to the alphaherpesvirus OBP and that initiation of HHV-6B DNA replication may resemble that of alphaherpesviruses.

Human herpesviruses 6A and 6B (HHV-6A and HHV-6B) are T-lymphotropic viruses that as a group are nearly ubiquitous, with primary infection occurring in most cases by 3 years of age (30, 48). The classification of HHV-6 isolates into the variants A and B (1) is based on differences in molecular and biologic properties (2, 4, 5, 15, 49, 58); HHV-6A has not been clearly associated with any clinical entity, while HHV-6B is an etiologic agent of roseola (exanthem subitum) and acute nonspecific febrile illness in infants (18, 45, 49, 59).

HHV-6A and HHV-6B genomes consist of an approximately 140-kb unique region and a pair of 10- to 13-kb directly repeated terminal elements, DR_L and DR_R (35, 37, 44). Analyses of HHV-6A and HHV-6B nucleotide and encoded protein sequences indicate that these viruses are genetically colinear with human cytomegalovirus (HCMV) over much of their genomes (10, 11, 20, 25, 33, 34, 41), but also that there are significant differences with HCMV with respect to genome size (161 to 170 kb for HHV-6 versus 230 kb for HCMV), genome structure (terminal direct repeats versus inverted repeats that mediate inversion events), G+C content (43% versus 56%), and the existence of unique genes and repeated sequences (37, 54).

We identified an HHV-6B open reading frame (ORF), CH6R, that encodes a homolog of the alphaherpesvirus origin-binding protein (OBP) (10). This observation is intriguing because examples of this gene were previously found only in alphaherpesviruses, although HCMV and Epstein-Barr virus do encode positional homologs (7, 9, 39, 53). On the basis of results described in this work, we have designated the HHV-6B CH6R protein as OBP_{H6B}. The herpes simplex virus type 1 (HSV-1) OBP (OBP_{H1}) is encoded by the UL9 gene and is one of the seven gene products essential for HSV-1 DNA replication (57). OBP_{H1} consists of a helicase domain and a DNA-binding domain (14, 38, 42, 55). OBP_{H6B} contains a well-conserved helicase motif, but the sequence has diverged in the region corresponding to the OBP_{H1} DNA-binding domain (10). The divergence suggested several possibilities concerning the function of this protein (10). First, it was possible that the divergent carboxyl region of OBP_{H6B} may still form a structural motif similar to those required for DNA binding by OBP_{H1}. Second, it was also possible that OBP_{H6B} may not exhibit sequence-specific DNA binding, and that the carboxyl region could be involved in the formation of a multisubunit complex with other viral and cellular proteins which confer sequence-specific DNA binding. Lastly, it was possible that the divergence reflects the evolution of an ancestral OBP toward recognition of divergent replication origin sequences as observed in EBV and HCMV *oriLyt* structures, which are complex and spread out over more than 1 kb (3, 26).

During analysis of other HHV-6B(Z29) DNA sequences, we identified a unique structure that includes two 195-bp AT-rich imperfect direct repeats and an adjacent GC-rich motif in the intergenic region between the major DNA-binding protein (MDBP) and HCMV UL69/HSV-1 ICP27 homologs (11). Because of these properties and because the region upstream

* Corresponding author. Mailing address: Centers for Disease Control and Prevention, Mailstop G18, 1600 Clifton Rd., Atlanta, GA 30333. Phone: (404) 639-2186. Fax: (404) 639-0049. Electronic mail address: php1@ciddvdl.em.cdc.gov.

† Present address: Department of Virology I, National Institute of Health, Tokyo 162, Japan.

‡ Present address: Central Dupont Central Research and Development, Experimental Station, Wilmington, DE 19808.

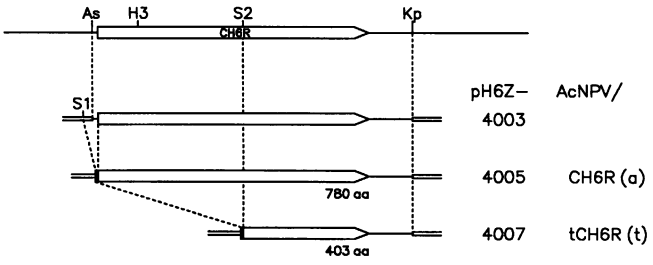

FIG. 1. OBP_{H6B}-expressing recombinant plasmids and baculoviruses. Procedures for construction of these plasmids are described in Materials and Methods. *Ase*I (As), *Hin*dIII (H3), *Kpn*I (Kp), *Sac*I (S1), and *Sac*II (S2) sites, the CH6R ORF (open bar), and TATAAAT (closed bar) are indicated. aa, amino acids.

of MDBP is known to function as an origin of lytic replication in HSV, varicella-zoster virus, and cytomegaloviruses (3, 51, 56), we hypothesized that this region might serve as an origin for lytic-phase DNA replication.

On the basis of these hypotheses and evidence, in this study we identified OBP_{H6B} transcripts in HHV-6B-infected cells, expressed OBP_{H6B} in two systems to test the possibility that OBP_{H6B} binds in a sequence-specific manner to the putative *ori* region, and found that the OBP_{H6B} DNA-binding sequences are similar in structure to origins of the lytic replication of alphaherpesviruses.

MATERIALS AND METHODS

Construction of plasmids. (i) pH6Z-4003. pH6Z-202 containing the HHV-6B strain Z29 *Hin*dIII D fragment (34) was digested with *Cla*I and *Kpn*I. The *Cla*I-*Kpn*I fragment containing the carboxyl region of the CH6R ORF was inserted between the *Cla*I and *Kpn*I sites of pBluescriptII KS(+) (Stratagene, La Jolla, Calif.) to construct pH6Z-4002. pH6Z-607 containing HHV-6B *Cla*I H fragment (34) was digested with *Ase*I, made blunt ended by treatment with T4 DNA polymerase, and then digested with *Cla*I. The *Ase*I-*Cla*I fragment containing the amino-terminal region of the CH6R ORF was cloned between the *Eco*RV and *Cla*I sites of pH6Z-4002, resulting in pH6Z-4003. pH6Z-4003 thus contains the entire CH6R ORF with 40 bp of HHV-6B noncoding sequence upstream of the first ATG codon and 371 bp downstream of the stop codon (Fig. 1).

(ii) pH6Z-4005. The *Sac*I-*Hin*dIII fragment of pH6Z-4003 was cloned between the *Sac*I and *Hin*dIII sites of M13mp18 (60). The resulting M13 plasmid (pH6Z-4023) was mutagenized by the method of Kunkel et al. (31), using a 45-nucleotide (nt) oligonucleotide, 5′-TTCTGAACCTGT TCTCCATATTTATAGAGCTCGAATTCGTAATC-3′, to replace the 5′ noncoding region of CH6R and the multiple cloning sites between the *Sac*I and *Eco*RV sites with the *Autographa californica* nuclear polyhedrosis virus (AcNPV) polyhedrin promoter sequence, TATAAAT. The resulting M13 plasmid (pH6Z-4024) was further mutagenized with an oligonucleotide, 5′-TTCCTTGGGAACTTGTTCA-3′, to destroy a *Kpn*I site in the ORF without altering the translated amino acid sequence (pH6Z-4025). Finally, the *Sac*I-*Hin*dIII fragment from pH6Z-4003 was replaced with the mutagenized *Sac*I-*Hin*dIII fragment of pH6Z-4025, resulting in pH6Z-4005 (Fig. 1).

(iii) pH6Z-4007. pH6Z-4007 was constructed by digestion of pH6Z-4003 with *Sac*I and *Sac*II and ligation of the digested DNA with a linker,

5′- CTATAAATATGGGC-3′
3′-TCGAGATATTTATACC-5′,

which contains the polyhedrin promoter and an in-frame ATG sequence (Fig. 1).

(iv) pH6Z-4041 and pH6Z-4043. pH6Z-4005 and pH6Z-4007 were digested with *Sac*I, made blunt ended by treatment with T4 DNA polymerase, and then digested with *Kpn*I. A plasmid vector containing the AcNPV polyhedrin gene, pAcDSM (47), was digested with *Pst*I, treated with T4 DNA polymerase, and then digested with *Kpn*I. The *Sac*I-*Kpn*I fragments of pH6Z-4005 and pH6Z-4007 were ligated with the digested pAcDSM, resulting in pH6Z-4041 and pH6Z-4043, respectively. These plasmids encode authentic and truncated forms of the HHV-6B CH6R protein with no additional amino acids derived from the polyhedrin ORF.

In vitro translation. Authentic and truncated forms of the CH6R protein were expressed in coupled transcription-translation reactions (T_NT reticulocyte lysate; Promega, Inc., Madison, Wis.) from plasmids pH6Z-4005 and pH6Z-4007. Under conditions recommended by the manufacturer, 1 μl of the reticulocyte lysate may contain 50 to 100 μg of total protein and 3 to 10 ng of the translated protein. ^{35}S-labeled, in vitro-translated products were mixed with an equal volume of loading buffer containing 6 M urea, 2% sodium dodecyl sulfate (SDS), and 1.8 M 2-mercaptoethanol and separated by SDS-polyacrylamide gel electrophoresis (PAGE) (32). Gels were treated with an intensifying agent (EN^3Hance; Dupont NEN, Boston, Mass.), dried, and then exposed to film (XAR-5; Kodak, Rochester, N.Y.).

Baculovirus expression. The recombinant baculoviruses AcNPV/CH6R and AcNPV/tCH6R were obtained by cotransfection of *Spodoptera frugiperda* Sf9 cells with AcNPV DNA plus pH6Z-4041 and pH6Z-4043, respectively. Procedures for transfection, selection of recombinant baculoviruses, and virus titration were as described previously (52). Sf9 and High Five (Invitrogen, San Diego, Calif.) insect cells were cultured in Hink's medium supplemented with 10% fetal calf serum and infected with recombinant or wild-type baculoviruses at a multiplicity of infection of 5 to 10 PFU per cell. After 60 to 70 h, cells were harvested and nuclear fractions were prepared as described previously (23). Nuclei were fractionated into a

TABLE 1. Oligonucleotides used for RT-PCR[a]

Name	Sequence	Coordinates
OBPFOR	5′-GTGACGGGTGTGTTTTCTAT-3′	5263–5282
OBPXREV	5′-TTCTCGAGCAACTTCACTAACGGAAAGG-3′	5560–5541
OBP probe	5′-GCCCTCACCGAGGCCTCGAGAGCAAGGCGG-3′	5473–5502
ACTINFOR	5′-TCATCACCATTGGCAATGAG-3′	2405–2424
ACTINREV	5′-CACTGTGTTGGCGTACAGGT-3′	2654–2635
ACTIN probe	5′-GGGCATGGAGTCCTGTGGCATCCACGAAAC-3′	2561–2590

[a] HHV-6B sequence coordinates are from reference 10, and actin sequences are from reference 40.

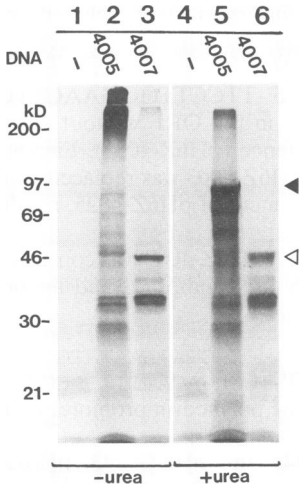

FIG. 2. In vitro expression of OBP_{H6B}. Transcription-translation reaction mixtures without added DNA (lanes 1 and 4) and programmed with pH6Z-4005 DNA (lanes 2 and 5) or with pH6Z-4007 DNA (lanes 3 and 6) were incubated in the presence of [^{35}S]methionine. Labeled protein was mixed with loading buffer without (lanes 1 to 3) or with (lanes 4 to 6) 6 M urea, analyzed by SDS-PAGE, and detected by fluorography. Expected bands corresponding to authentic and truncated sizes of the protein are indicated with closed and open arrowheads, respectively.

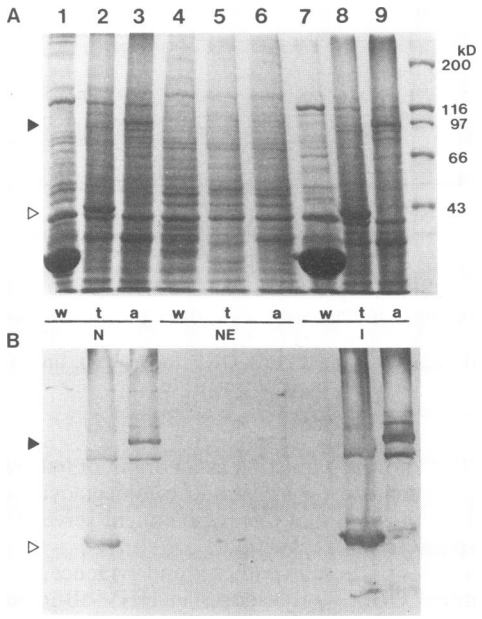

FIG. 3. Baculovirus expression of OBP_{H6B} in insect cells. High Five insect cells were infected with AcNPV (w; lanes 1, 4, and 7), AcNPV/tCH6R (t; lanes 2, 5, and 8), and AcNPV/CH6R (a; lanes 3, 6, and 9) at a multiplicity of infection of 5 to 10. After 65 h, cells were harvested and nuclear fractions were prepared, extracted in the presence of 2 M NaCl and 0.5% Nonidet P-40, and then cleared by centrifugation. Proteins in the nuclear fraction (N; lanes 1 to 3), nuclear extract (NE; lanes 4 to 6), and insoluble fraction (I; lanes 7 to 9) were separated in two SDS-polyacrylamide gels. One gel was stained with Coomassie brilliant blue (A), and the other was used for immunoblotting with antiserum against the A9-3 oligopeptide (B). Arrowheads are as described in the legend to Fig. 2.

soluble nuclear extract and an insoluble fraction by centrifugation (40,000 × *g*, 30 min) after incubation in the presence of 2 M NaCl and 0.5% Nonidet P-40 at 4°C for 30 min.

Preparation of antiserum. Oligopeptides A9-1 (ITERQIN NRKV) and A9-3 (SRARRKQTIYKLQGS), corresponding to OBP_{H6B} amino acid residues 106 to 116 and 742 to 756, respectively, were conjugated with keyhole limpet hemocyanin by using an Imject EDC immunogen kit (Pierce Inc., Rockford, Ill.). The conjugates were mixed with an adjuvant (Titer-Max; CytRx Co., Norcross, Ga.) and used to immunize female New Zealand White rabbits.

Immunoblotting. Proteins were mixed with urea-containing loading buffer, sonicated three times for 30 s each in a cup horn sonicator (output control 4, duty cycle 50%; model W-375; Heat Systems-Ultrasonic, Inc., Farmingdale, N.Y.), separated by SDS-PAGE, and then transferred onto nitrocellulose filters (BA85; Schleicher & Schuell, Keene, N.H.) in a transfer buffer containing 25 mM Tris base, 250 mM glycine, and 20% methanol. The blots were incubated for 3 h in phosphate-buffered saline (PBS) containing 2% skim milk and then for 2 h in PBS containing 1% skim milk and rabbit antiserum. After two 10-min washes in PBS containing 0.05% Tween 20, the blots were reacted with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad, New York, N.Y.) in PBS and then developed with *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Bio-Rad) according to the vendor's protocol.

Gel mobility shift assays. Nineteen sets of double-stranded oligonucleotides spanning a 644-bp region in the intergenic region between the MDBP and HCMV UL69/HSV-1 ICP27 homologs were synthesized as follows. Each set of oligonucleotides consisted of two 30-nt-long (sets 13 to 19) or 35-nt-long (sets 1 to 12) single-stranded oligonucleotides (model 380 DNA synthesizer; Applied Biosystems, Foster City, Calif.) with 15-nt (sets 13 to 19) or 20-nt (sets 1 to 12) complementary sequences. After annealing, each set of oligonucleotides was incubated with T4 DNA polymerase in the presence of [^{32}P]dCTP and [^{32}P]dATP, and then an excess of unlabeled dCTP and dATP was added to complete the repair reaction. Free nucleotides were eliminated by size exclusion chromatography (Push column; Stratagene).

Next, 0.3 pmol (approximately 5 × 10^4 cpm) of ^{32}P-labeled 45-bp (sets 13 to 19) or 55-bp (sets 1 to 12) DNA was incubated at 20°C for 20 min in 20 μl of reaction buffer with 1 μl of transcription-translation lysate prepared without DNA or with plasmid DNA. The reaction buffer consisted of 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.6), 4 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 60 μg of bovine serum albumin per ml, 12% glycerol, 5 μg of salmon testes DNA per ml, and a cocktail of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.24 trypsin inhibitor units of aprotinin per ml, 10 ng of pepstatin A per ml, and 10 ng of leupeptin per ml). DNA-protein complexes were separated in 4% polyacrylamide gels (80:1) by using a low-ionic-strength electrophoresis buffer (6.725 mM Tris-HCl, 3.3 mM sodium acetate, 1 mM EDTA [pH 7.6]) at 4°C.

Southwestern (DNA-protein) blotting. Southwestern blotting was performed by the method described previously (19), with minor modifications. Insoluble fractions containing baculovirus-expressed full-length and truncated forms of OBP_{H6B} were separated by SDS-PAGE in 7.5% gels, and proteins were transferred to nitrocellulose. Blots were incubated in 20 mM Tris-HCl (pH 7.6)–50 mM NaCl–1 mM EDTA–1 mM DTT–5% skim milk for 1 h and washed twice in the same

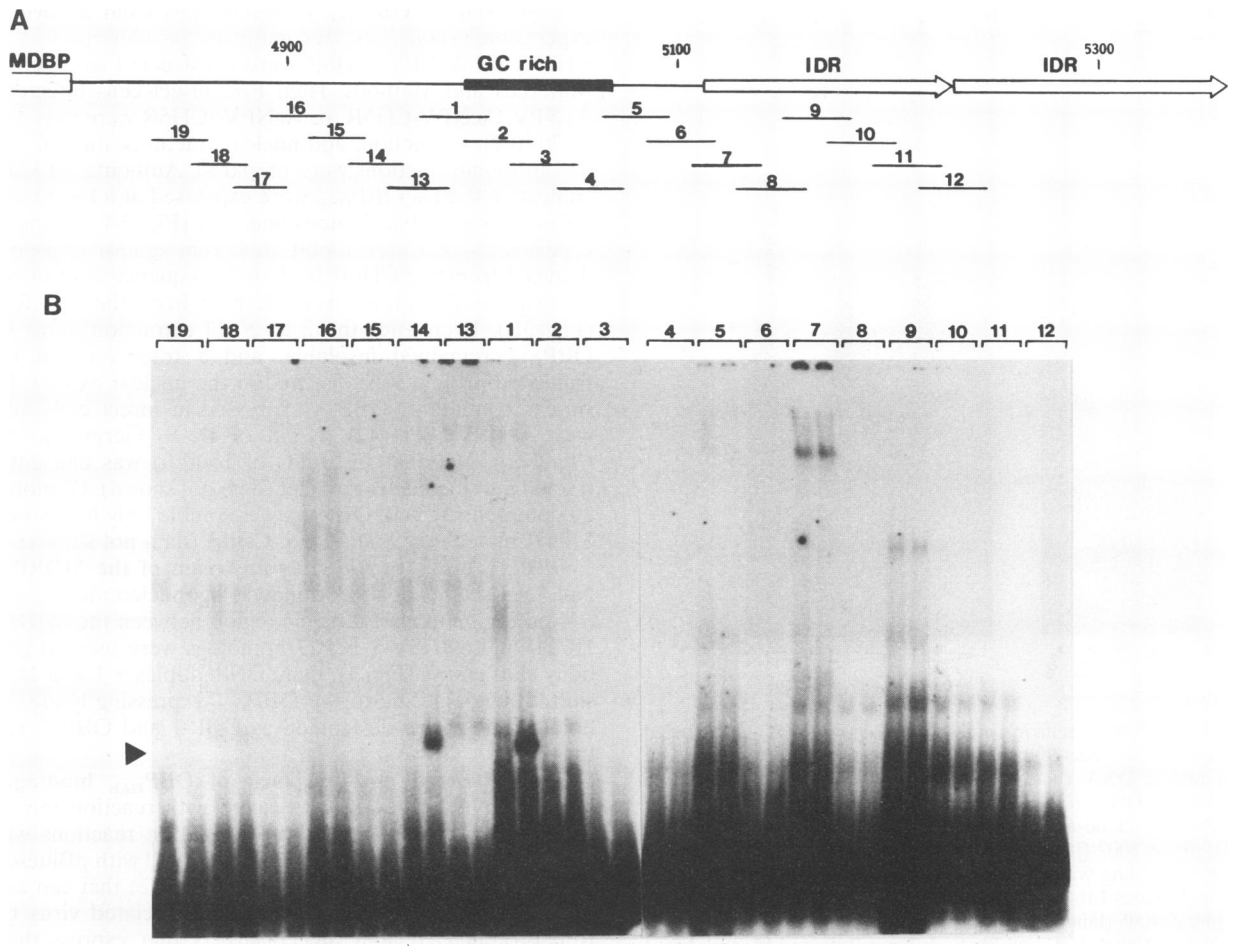

FIG. 4. Gel mobility shift assay to identify OBP_{H6B} binding sites. (A) Schematic diagrams of the upstream region of the MDBP gene, including the GC-rich region and imperfect direct repeats (IDR) (11), and 19 sets of double-stranded oligonucleotides used for the gel mobility shift assay. (B) Gel mobility shift assay. ^{32}P-labeled oligonucleotides were reacted with transcription-translation lysate prepared without DNA (left lanes for each [^{32}P]DNA) or with pH6Z-4007 DNA (right lanes) as described in Materials and Methods. Mobility shifts specific to the lysates programmed with pH6Z-4007 are indicated by the arrowhead.

buffer without skim milk for 5 min. Blots were then incubated in 7 M guanidine-HCl–50 mM Tris-HCl (pH 7.6)–2 mM EDTA–10 mM DTT–0.25% skim milk for 1 h, washed with 20 mM Tris-HCl (pH 7.6)–50 mM NaCl–1 mM EDTA–1 mM DTT–0.1% Nonidet P-40–0.25% skim milk, and then incubated in the same buffer at 4°C overnight. Blots were incubated with 3 pmol of ^{32}P-labeled DNA duplex in 2.5 ml of 20 mM Tris-HCl (pH 7.6)–50 mM NaCl–1 mM EDTA buffer with either 2.5 μg of poly(dI-dC)-poly(dI-dC) or 0.5 μg of salmon testes DNA at room temperature for 3 h and washed twice for 5 min in the same buffer without carrier or labeled DNA.

Reverse transcription PCR (RT-PCR). The oligonucleotide primers used for amplification and probes used for detection of transcripts near the 3′ end of the CH6R ORF and human β-actin transcripts are described in Table 1.

Molt-3 cells were infected by mixing Molt-3-adapted HHV-6B(Z29) with uninfected cells at a 1:10 ratio. Three days after infection, total RNA was extracted from cells with RNAzol B (CINNA/BIOTECX Laboratories International Inc., Friendswood, Tex.) according to the manufacturer's protocol. mRNA was purified from the extracted total RNA with a QuickPrep Micro mRNA purification kit (Pharmacia, Piscataway, N.J.). Eluted mRNA was concentrated by spin dialysis with a Centricon-30 (Amicon, Beverly, Mass.) by centrifugation at 5,000 × *g* for 30 min. For each RT-PCR, mRNA from 3×10^5 cells was desiccated in a vacuum centrifuge and resuspended in 8 μl of a solution containing 2 mM magnesium chloride, 5,000 U of RNasin RNase inhibitor (Promega) per ml, and 125 U of RQ1 RNase-free DNase (Promega) per ml. Samples were incubated for 1 h at 37°C, overlaid with 1 drop of mineral oil, incubated for 5 min at 90°C, and then immediately placed on ice. RT-PCR assays were performed on the products of the DNase reactions by using a GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.) as described in the kit protocol with the exceptions of a 55°C PCR annealing temperature and final PCR primer concentrations of 0.2 μM each. Thermal cycling (DNA Thermal Cycler; Perkin-Elmer Cetus) parameters were 2 min at 95°C for 1 cycle, 1 min at 95°C and 1 min at 55°C for 35 cycles, and 7 min at 60°C for 1 cycle. Twenty microliters of each RT-PCR product was separated on an 8.0% polyacrylamide gel and blotted to a nylon membrane (Nytran; Schleicher & Schuell) by semidry electrical transfer (Trans-blot SD semidry transfer cell; Bio-Rad). Probe oligonucleotides were 5′ radiolabeled by using polynucleotide kinase (KinAce-It; Stratagene), and hybridizations were in 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–0.1% sodium

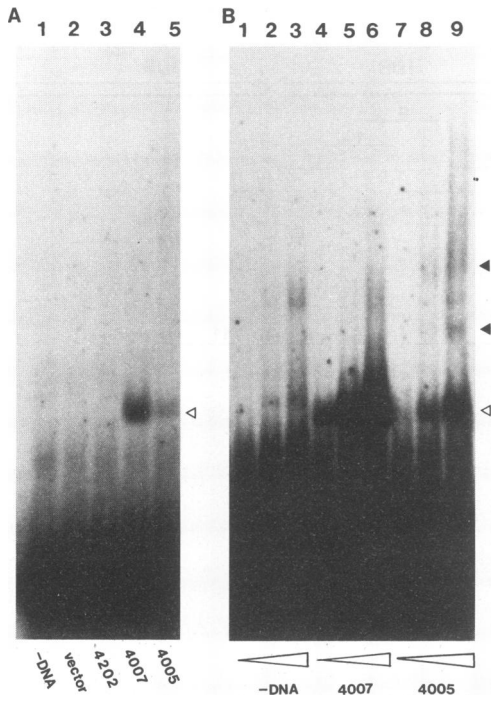

FIG. 5. Binding specificity and dose response. (A) ^{32}P-labeled DNA duplex 1 was reacted with 1 μl of transcription-translation lysate without added DNA (lane 1) or programmed with pBluescriptII KS(+) vector DNA (lane 2), pH6Z-4202, which can express the HHV-6B(Z29) adeno-associated virus type 2 Rep protein homolog (lane 3), pH6Z-4007 (lane 4), and pH6Z-4005 (lane 5). (B) The dose response of binding with the transcription-translation lysate without added DNA (lanes 1 to 3) or programmed with pH6Z-4007 (lanes 4 to 6) and pH6Z-4005 (lanes 7 to 9) was tested by changing the amount of lysate: 0.25 μl (lanes 1, 4, and 7), 1 μl (lanes 2, 5, and 8), and 2.5 μl (lanes 3, 6, and 9). Specific mobility shifts are indicated by arrowheads.

pyrophosphate, 200 μg of heparin per ml–0.2% SDS at 65°C. Final washes were in 2× SSC–0.1% SDS at 65°C.

RESULTS

Expression of OBP_{H6B} in vitro. Authentic and truncated forms of OBP_{H6B} were expressed in vitro in coupled transcription-translation reactions from plasmids pH6Z-4005 (Fig. 2, lanes 2 and 5) and pH6Z-4007 (lanes 3 and 6) in the presence of [^{35}S]methionine. Labeled protein was separated by SDS-PAGE. The apparent molecular sizes of the authentic and truncated forms of the protein, 95 kDa and 45 kDa, were consistent with the predicted sizes of 90 and 46 kDa, respectively. In addition to these expected products, some small products that might be products of proteolysis or initiation of translation from internal AUG sequences were present. Of these fragments, the 33- and 35-kDa species were observed in both pH6Z-4005 and pH6Z-4007 lysates, suggesting that these species were derived from the carboxyl region of OBP_{H6B}. The 45-kDa protein expressed from pH6Z-4007 but not the 95-kDa species from pH6Z-4005 was visible without addition of urea to the SDS-PAGE sample loading buffer (lanes 2 and 3). Addition of 50% (vol/vol) ethylene glycol to the loading buffer gave results similar to those obtained with 6 M urea. These results suggest that OBP_{H6B} has a strongly hydrophobic character and that this property is associated with the amino-terminal domain of the molecule.

Expression of OBP_{H6B} in insect cells from a baculovirus expression vector. The recombinant baculoviruses AcNPV/CH6R and AcNPV/tCH6R were constructed as described in Materials and Methods. High Five insect cells infected with AcNPV, AcNPV/CH6R, or AcNPV/tCH6R were harvested 60 to 70 h after infection, and nuclear fractions, nuclear extract, and insoluble fractions were prepared. Authentic 95-kDa and truncated 45-kDa OBP_{H6B} were expressed at a level visible in a Coomassie brilliant blue-stained gel (Fig. 3A). Immunoblot experiments that used rabbit antiserum against oligopeptides deduced from the CH6R nucleotide sequence confirmed that the expressed proteins were derived from the CH6R ORF (Fig. 3B). Even after the 2 M NaCl extraction, most of the OBP_{H6B} remained insoluble, and a trace amount of the truncated protein was observed in the nuclear extract. Under similar conditions, OBP_{H1} expressed in insect cells infected with AcNPV/UL9 (42) (a gift of D. S. Fierer and M. D. Challberg, National Institutes of Health) was efficiently extracted into the nuclear extract (data not shown). Of more than 20 detergents tested, OBP_{H6B} was soluble only in Zwittergent 3-14 (Calbiochem, San Diego, Calif.) (data not shown).

OBP_{H6B} binds to two sites upstream of the MDBP gene. Nineteen sets of double-stranded oligonucleotides covering a 644-bp region in an intergenic region between the MDBP and HCMV UL69/HSV-1 ICP27 homologs were used in gel mobility shift assays (Fig. 4). Only DNA duplexes 1 and 14 had a shifted band specific to the OBP_{H6B}-expressing lysate. These binding sites were designated as OBP-1 and OBP-2, respectively.

Specificity and dose response of OBP_{H6B} binding. ^{32}P-labeled DNA duplex 1 was reacted with reaction mix either from the coupled transcription-translation reactions without added DNA or from reactions programmed with pBluescriptII KS(+) vector DNA, a plasmid (pH6Z-4202) that can express the HHV-6B homolog of the adeno-associated virus type 2 Rep protein (28), and the constructs that express the full-length and truncated forms of OBP_{H6B} (pH6Z-4005 and pH6Z-4007, respectively) (Fig. 5A). This experiment confirmed that the binding is specific to the CH6R-expressing lysates (lanes 4 and 5).

The dose response of binding with the transcription-translation lysate was shown by varying the amount of lysate used in the gel mobility shift assay (Fig. 5B). When the amount of lysate was increased, the lysate programmed with the plasmid encoding full-length OBP_{H6B} (pH6Z-4005) gave at least two specific bands (closed arrowheads in lane 9) in addition to the band with the same size as that observed in the truncated form, suggesting the pH6Z-4005-programmed lysate contains different forms of OBP_{H6B}. One possibility is that the aggregated authentic form of the protein shown in Fig. 3 could not bind efficiently to the DNA and that the observed shifts were mainly caused by the small carboxy terminal-derived fragments.

Defining the OBP-1 boundaries. Gel mobility shift assays with 31-bp-long ^{32}P-labeled DNA duplexes 1A to 1D were performed under the same conditions as described for Fig. 4. These four DNA duplexes span the 55-bp region contained in duplex 1 in an overlapping manner. DNA duplexes 1B and 1C, but not 1A and 1D, bound to OBP_{H6B} (Fig. 6A and C).

Sixteen- and eightyfold-greater amounts of competitor DNAs (1A to 1G) were added to reactions that tested for competition against binding of ^{32}P-labeled DNA duplex 1. DNA duplexes 1B, 1C, and 1F competed with the binding, indicating that the 23-bp DNA region corresponding to DNA duplex 1F encompasses the most narrowly defined OBP-1 binding region (Fig. 6B and C).

A 36-bp-long DNA fragment containing HSV-1 *ori*$_S$ box I

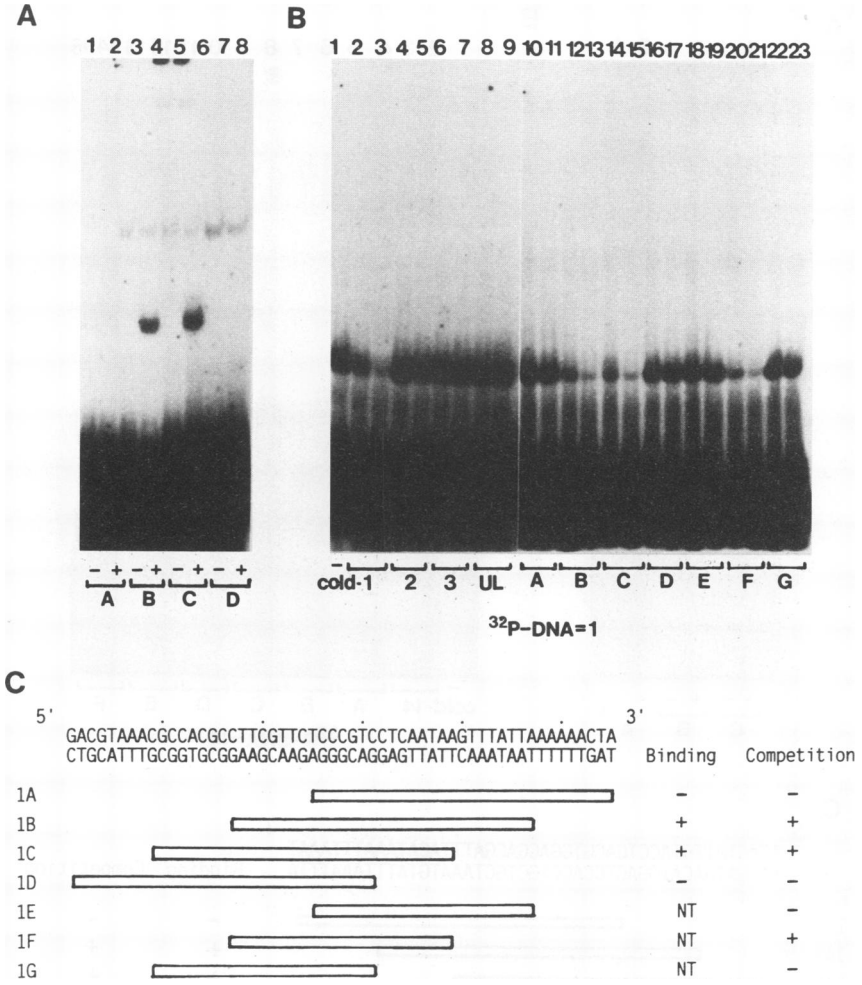

FIG. 6. Defining the OBP-1 sequence. (A) Gel mobility shift assays with 31-bp ^{32}P-labeled DNA duplexes 1A (lanes 1 and 2), 1B (lanes 3 and 4), 1C (lanes 5 and 6), and 1D (lanes 7 and 8) were performed under the conditions described for Fig. 4. Lysates were prepared without added DNA (lanes 1, 3, 5, and 7) and after being programmed with a plasmid that can direct the synthesis of the carboxy-terminal portion of OBP_{H6B} (pH6Z-4007) (lanes 2, 4, 6, and 8). (B) Competition of unlabeled DNA against binding of ^{32}P-labeled DNA duplex 1. Sixteen- and eightyfold amounts of competitor DNAs were used. Oligoduplexes 1 to 3 (lanes 2 to 7) depicted in Fig. 4, 1A to 1F (lanes 10 to 23) depicted in panel C, and a 36-bp-long DNA fragment containing HSV-1 ori_S box I (UL) were used as competitor DNAs. (C) Summary of binding and competitor assay results. +, signs for the binding assay indicate detection of a mobility shift, and those for the competitor assay indicate competition with the labeled DNA. NT, not tested.

(UL) was prepared by the method of Elias and Lehman (22) and used as competitor DNA as well. It did not show any competition against binding of ^{32}P-labeled DNA duplex 1 (Fig. 6B, lanes 8 and 9), indicating that the DNA sequence recognized by OBP_{H6B} is HHV-6B specific. In addition, no mobility shift was seen when a nuclear extract containing the HSV-1 UL9 protein prepared from insect cells infected with AcNPV/UL9 was reacted with DNA duplex 1. Furthermore, DNA duplex 1 did not compete with the binding of OBP_{H1} to the HSV-1 box I sequence (data not shown).

Defining the OBP-2 boundaries. The OBP-2 sequence was defined in gel shift assays with three 30-bp-long, ^{32}P-labeled DNA duplexes, 14A, 14B, and 14C. DNA duplexes 14B and 14C, but not 14A, bound to OBP_{H6B} (Fig. 7A and C). Competition assays were performed with excess 14A to 14G DNA; DNA duplexes 14B, 14C, and 14E showed competition (Fig. 7B and C). These results indicate that the 23-bp DNA region corresponding to 14E DNA is the most narrowly defined OBP-2 sequence.

Southwestern blotting with baculovirus-expressed OBP_{H6B}. Insoluble fractions containing baculovirus-expressed authentic and truncated forms of OBP_{H6B} (described in Fig. 3) were used in a Southwestern blotting assay. ^{32}P-labeled DNA duplex 1 was reacted with the blots in the presence of a 500-fold-greater mass of poly(dI-dC)-poly(dI-dC) (Fig. 8, lanes 1 to 3) or 100-fold more salmon testes DNA (lanes 4 to 6). DNA duplex 1 bound to both the authentic and truncated forms of the baculovirus-expressed OBP_{H6B} under both conditions (Fig. 8). The signal from the authentic form of OBP_{H6B} was weaker than that of the truncated form. This difference can be accounted for because all lanes were loaded with material from similar numbers of cells, and the level of expression of authentic OBP_{H6B} was smaller than that of the truncated form (Fig. 3A, lanes 8 and 9). DNA duplex 1B, but neither duplex 1A nor 1D, bound to OBP_{H6B} under the same conditions (data not shown).

OBP-1 and OBP-2 are similar to alphaherpesvirus *ori* sequences. Figure 9 depicts a comparative alignment of the

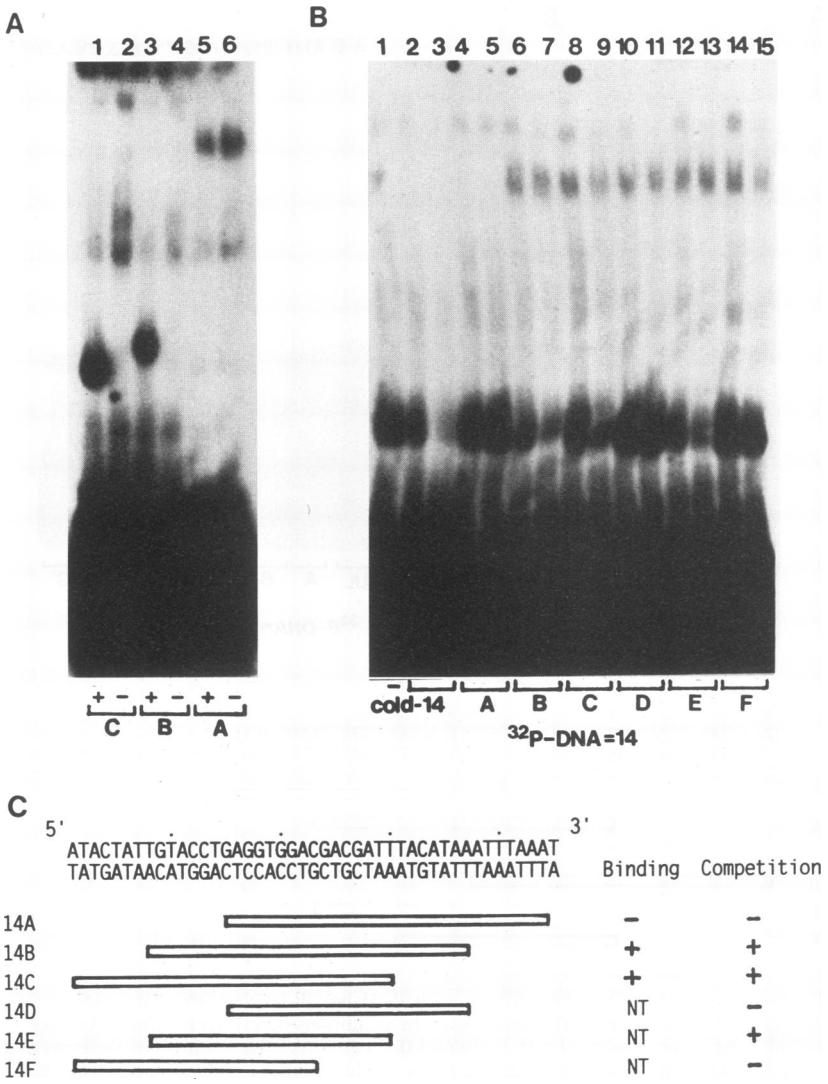

FIG. 7. Defining the OBP-2 sequence. (A) Gel mobility shift assays with 30-bp ^{32}P-labeled DNA duplexes 14A to 14C were performed as described in the legend to Fig. 4. (B) Competition of unlabeled DNA against binding of ^{32}P-labeled DNA duplex 14. Sixteen- and eightyfold amounts of competitor DNAs were used. Oligoduplexes 14 and 14A to 14F were used as competitors. (C) Summary of binding and competition assay results (as described in the legend to Fig. 6).

region including OBP-1 and OBP-2 with *ori* sequences of several alphaherpesviruses: HSV-1, HSV-2, varicella-zoster virus, equine herpes virus 1, pseudorabies virus, and Marek's disease virus. The following similarities between the OBP_{H6B} binding region and the alphaherpesvirus origins are evident in the alignment. (i) OBP-1 has seven identical residues within the 10-bp core sequence for OBP_{H1} binding, YGYTCGCACT (Y equals pyrimidine nucleotide) (13, 21, 27). OBP-2 also has similarity with this sequence. (ii) The binding regions are separated by an AT-rich sequence. (iii) The binding regions are part of an element of dyad symmetry that has the potential of forming a stem-loop structure.

To test the possibility that HHV-6 genomes contain other sequences similar to that of OBP-1 or OBP-2, all of the HHV-6A and HHV-6B nucleotide sequences in GenBank release 78, as well as additional unpublished sequences (12), were examined for the presence of similar sequences by using the Genetics Computer Group program Findpatterns. With query sequences consisting of CGTTCTCCCGTCCTG (OBP-1) and GAGGTGGACGACGAT (OBP-2), eight sequences that have three or fewer mismatches were found. However, most of the sequences have three mismatches in 15 bp and are found within ORFs. Moreover, none of the eight loci has overall structural similarity with the OBP-1 and OBP-2 regions (data not shown).

The segment of OBP-1 corresponding to the 10-bp HSV-1 core binding sequence is essential for OBP_{H6B} binding. Mutations in the central CGC sequence of the OBP_{H1} core binding sequence, YGYTCGCACT, severely reduced its binding to OBP_{H1} (27). Therefore, we tested the effects of mutations in the OBP-1 CTC sequence that corresponds to the HSV-1 CGC sequence on OBP_{H6B} binding (Fig. 10). DNA duplexes 1F-M5, 1F-M6, and 1F-M7 have the sequences GTC, CGC, and CTG, respectively, in place of the CTC sequence in the 1F DNA duplex. No competition was seen with 1F-M7, and very limited competition was seen with 1F-M5 and 1F-M6,

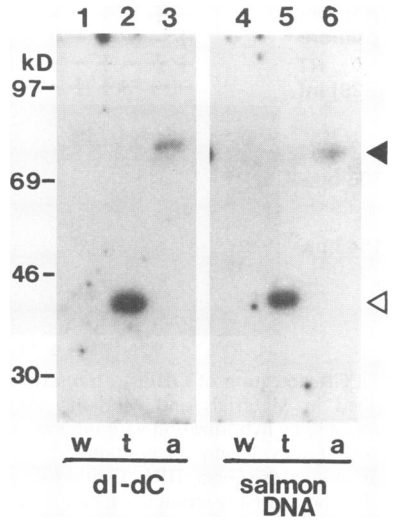

FIG. 8. Southwestern blotting with baculovirus-expressed OBP_{H6B}. Insoluble nuclear fractions prepared from cells infected with AcNPV (w; lanes 1 and 4), AcNPV/tCH6R (t; lanes 2 and 5), or AcNPV/CH6R (a; lanes 3 and 6) were separated by SDS-PAGE in 7.5% gels, and the proteins were transferred to nitrocellulose. The blots were analyzed by the Southwestern blotting method with ^{32}P-labeled DNA duplex 1 as the probe. Poly(dI-dC)-poly(dI-dC) (lanes 1 to 3) or salmon testes DNA (lanes 4 to 6) was used as the competitor DNA. Closed and open arrowheads indicate bands corresponding to authentic and truncated forms of OBP_{H6B}, respectively.

1F-M6 being the stronger competitor. Under similar conditions, duplex 1F nearly quantitatively abolished the binding.

OBP_{H6B} expression in HHV-6B(Z29)-infected cells. mRNA was harvested from HHV-6B(Z29)-infected and uninfected Molt-3 cells 3 days after infection. OBP_{H6B}-specific amplification by RT-PCR was obtained only from mRNA isolated from infected cells (Fig. 11). No amplification was seen if the reverse transcription step was omitted, and actin transcripts were detected in all mRNA preparations. The kinetic properties of OBP_{H6B} transcription are under further investigation.

We also attempted to find direct evidence for expression of OBP_{H6B} in HHV-6B-infected lymphocytes by the gel mobility shift assay and by immunoblotting with two antisera against the protein. Although some experiments gave positive signals, improvements in sensitivity and signal-to-noise ratios will be required to provide conclusive results (data not shown).

DISCUSSION

In this study, we detected transcripts from the gene encoding OBP_{H6B}, the HHV-6B homolog of the alphaherpesvirus OBP, expressed OBP_{H6B} in two expression systems, and found that the expressed proteins bound to two sites, designated OBP-1 and OBP-2, in the putative HHV-6B *ori* region. We defined the minimum OBP-1 and OBP-2 sequences to within 23 bp each. Interestingly, OBP-1 and OBP-2 share sequence homology with HSV-1 *ori* boxes I and II, which are known as the DNA-binding sites of the HSV-1 UL9 protein (22, 36). Moreover, OBP-1 and OBP-2 are separated by an AT-rich sequence and are part of an imperfect dyad symmetry structure. Although there is a difference in the length of the AT-rich sequence, the basic structure is similar to those described for several alphaherpesviruses.

In addition to the similarity in the binding site sequences and structure, the following observations strongly support the idea that OBP_{H6B} is actually an HHV-6B OBP and functions in a manner similar to OBP_{H1}. (i) The binding sites are located upstream of the MDBP gene, a region that contains an origin of lytic replication in HSV-1 (56). (ii) Gene order in the region encoding OBP_{H6B} is conserved among other herpesviruses (HCMV UL98-UL105, HSV-1 UL12-UL5, EBV, BGLF5-BBLF4), and the position of the CH6R gene corresponds to that of the HSV-1 UL9 gene (10). (iii) The OBP_{H6B} amino acid sequence contains a helicase motif that is essential for HSV-1 DNA replication (38). (iv) The OBP_{H6B} DNA-binding domain is in the carboxy-terminal region of the molecule, as for OBP_{H1}.

Two other recent observations are related to this study. First, Dewhurst et al. (16) showed that a sequence segment that spans the OBP-1 and OBP-2 region functions in transient DNA replication assays as an origin in cells infected with HHV-6B. This observation supports the idea that OBP-1, OBP-2, and OBP_{H6B} are involved in HHV-6B DNA replication. Three sequence alterations in OBP-1 that had a negative effect on OBP_{H6B} binding (M5, M6, and M7; Fig. 10) also reduced the ability of plasmids to be replicated in the transient replication assay (17). Further studies will be required to determine whether the *ori* function observed in the transient DNA replication assay is also dependent on OBP-2. Further confirmation of a role for OBP_{H6B} in the initiation of lytic DNA replication will require the establishment of a transient complementation assay as was done for HSV-1 (57), HCMV (43), and EBV (24). Second, Stamey et al. (50) found gene amplification of the putative *ori* region in some laboratory-passaged HHV-6B virus stocks. The amplification may be

FIG. 9. Sequence comparison of OBP_{H6B}-binding sites with alphaherpesvirus *ori* sequences. The nucleotide sequence of the region that contains OBP-1 and OBP-2 was compared with *ori* sequences from several alphaherpesviruses (adapted from references 6, 8, and 29). HSV-1 S, HSV-1 ori_S; HSV1 L, HSV-1 ori_L; HSV-2 S, HSV-2 ori_S; HSV2 L, HSV-2 ori_L; EHV1 S, equine herpesvirus 1 ori_S; PrV, pseudorabies virus *ori*; VZV, varicella-zoster virus ori_S; MDV, Marek's disease virus *ori*. Residues identical to those found in the boxes I and II are in boldface. Horizontal arrows indicate a structure with dyad symmetry that is present in the HHV-6B region. I, II, and III, Box I, II, and III of HSV-1 ori_S (22, 36).

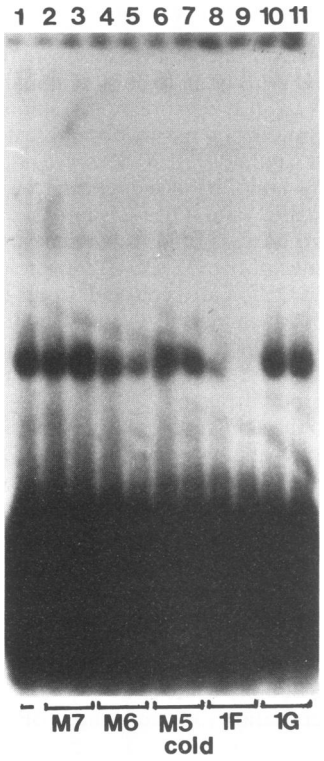

FIG. 10. Gel mobility shift assay with OBP-1 mutants. Competition assays were performed with DNA duplex 1F (lanes 8 and 9) and three DNA duplexes, 1F-M5 (lanes 6 and 7), 1F-M6 (lanes 4 and 5), and 1F-M7 (lanes 2 and 3), containing the indicated alterations:

```
1F     5'-CTTCGTTCTCCCGTCCTCAATAA-3'
1F-M5           G
1F-M6            G
1F-M7             G
```

somehow associated with OBP_{H6B}-dependent DNA replication.

An OBP_{H1} binding sequence was defined to a consensus sequence, YGYTCGCACT (13, 21, 27), and mutations in a central CGC sequence of the boxes severely reduced DNA-binding activity (27). We found that the corresponding three bases within OBP-1 are essential for OBP_{H6B} binding. Although an alteration of T to G in 1F-M6 makes the OBP-1 sequence more similar to the HSV-1 box I sequence, the alteration reduced the binding efficiency. Moreover, the HSV-1 box I sequence did not compete with the OBP_{H6B} binding (Fig. 6B). This binding site diversity may explain the lack of sequence similarity between the carboxy-terminal domains of OBP_{H6B} and the OBP of alphaherpesviruses. The binding affinity of OBP_{H1} to ori_S box I is higher than to box II (22). Although our experiments were not quantitative, there was no obvious difference in binding affinity between OBP-1 and OBP-2 (Fig. 4). Further detailed analysis with mutagenized DNA fragments will be required to clearly define the HHV-6B-specific recognition sequence.

Finally, we demonstrated that the carboxyl-terminal 403 amino acids were sufficient for the DNA-binding activity. The carboxy-terminal domain of OBP_{H1} spanning amino acid residues 564 to 832 contains the ori_S-specific DNA-binding domain. Although this domain of OBP_{H1} contains a pseudo-leucine zipper motif (14), the corresponding region of OBP_{H6B} does not. Further studies to map the minimal DNA-binding

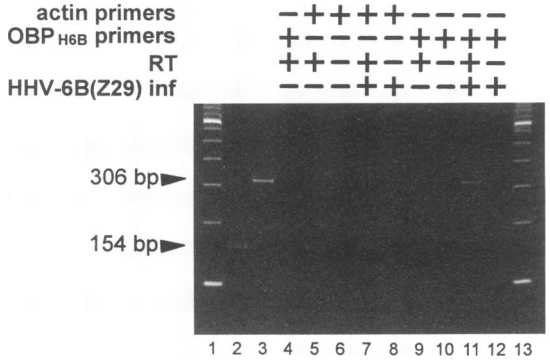

FIG. 11. RT-PCR detection of OBP_{H6B} transcripts. RT-PCR was done as described in Materials and Methods. Amplification was specific, as ascertained by hybridization with internal probe sequences (data not shown). Lanes 1 and 13 contain 100-bp DNA Ladder molecular weight standards (Gibco BRL, Gaithersburg, Md.). Lane 2 contains the PCR product of a reaction using human β-actin sequences as primers and Molt-3 cell cDNA as a template. Lane 3 contains the PCR product of a reaction using OBP_{H6B} sequences as primers and a nucleocapsid preparation of HHV-6B(Z29) DNA as a template. Lanes 4 to 12 are RT-PCRs performed under the indicated conditions: reverse transcriptase (RT); uninfected (−) or HHV-6B(Z29)-infected [HHV-6B(Z29) inf]. (+) Molt-3 cells.

domain and to compare this domain with that of OBP_{H1} may provide some insight to understanding the *ori*-binding motif. Moreover, analysis of the aggregation observed in the absence of urea may provide a key to understanding the higher-order structure of OBP_{H6B}. Both OBP_{H1} and OBP_{H6B} contain a leucine zipper motif near their amino termini, which may contribute to higher-order protein-protein interactions (10).

Our results suggest that the mechanism of HHV-6B DNA replication initiation resembles that of alphaherpesviruses and may differ from that of HCMV, a betaherpesvirus. Given the classification of HHV-6 as a betaherpesvirus (46), the evolution of these initiation mechanisms will be interesting subjects for further studies.

ACKNOWLEDGMENTS

We thank Daniel Fierer and Mark Challberg (National Institutes of Health) for providing HSV-1 UL9-expressing baculoviruses and Stephen Dewhurst for generously sharing data prior to publication. We also thank Demetrio Sánchez-Martínez for advice on baculovirus expression, D. Spensar and the CDC animal facility for assistance with the immunization of the rabbits, the CDC Biotechnology Core Facility for synthesis of oligopeptides and oligonucleotides, and Kathleen Kite-Powell, Dawn Ellis, and Jodi Black for propagation of HHV-6B.

N.I. was partially supported by the Japanese Agency of Science and Technology. J.C.R. was supported by a CDC/National Research Council postdoctoral fellowship.

REFERENCES

1. **Ablashi, D., H. Agut, Z. Berneman, G. Campadelli-Fiume, D. Carrigan, L. Ceccerini-Nelli, B. Chandran, S. Chou, H. Collandre, R. Cone, T. Dambaugh, S. Dewhurst, D. DiLuca, L. Foa-Tomasi, B. Fleckenstein, N. Frenkel, R. Gallo, U. Gompels, C. Hall, M. Jones, G. Lawrence, M. Martin, L. Montagnier, F. Neipel, J. Nicholas, P. Pellett, A. Razzaque, G. Torrelli, B. Thomson, S. Salahuddin, L. Wyatt, and K. Yamanishi.** 1993. Human herpesvirus-6 strain groups: a nomenclature. Arch. Virol. **129**:363–366.
2. **Ablashi, D. V., N. Balachandran, S. F. Josephs, C. L. Hung, G. R. F. Krueger, B. Kramarsky, S. Z. Salahuddin, and R. C. Gallo.** 1991. Genomic polymorphism, growth properties, and immunologic variations in human herpesvirus-6 isolates. Virology **184**:545–552.

3. **Anders, D. G., M. A. Kacica, G. Pari, and S. M. Punturieri.** 1992. Boundaries and structure of human cytomegalovirus *oriLyt*, a complex origin for lytic-phase DNA replication. J. Virol. **66**:3373–3384.
4. **Aubin, J. T., H. Agut, H. Collandre, K. Yamanishi, B. Chandran, L. Montagnier, and J. M. Huraux.** 1993. Antigenic and genetic differentiation of the two putative types of human herpes virus 6. J. Virol. Methods **41**:223–234.
5. **Aubin, J. T., H. Collandre, D. Candotti, D. Ingrand, C. Rouzioux, M. Burgard, S. Richard, J. M. Huraux, and H. Agut.** 1991. Several groups among human herpesvirus 6 strains can be distinguished by Southern blotting and polymerase chain reaction. J. Clin. Microbiol. **29**:367–372.
6. **Baumann, R. P., V. R. R. Yalamanchili, and D. J. O'Callaghan.** 1989. Functional mapping and DNA sequence of an equine herpesvirus 1 origin of replication. J. Virol. **63**:1275–1283.
7. **Buckmaster, A. E., S. D. Scott, M. J. Sanderson, M. E. G. Boursnell, N. L. J. Ross, and M. M. Binns.** 1988. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implications for herpesvirus classification. J. Gen. Virol. **69**:2033–2042.
8. **Camp, H. S., P. M. Coussens, and R. F. Silva.** 1991. Cloning, sequencing, and functional analysis of a Marek's disease virus origin of DNA replication. J. Virol. **65**:6320–6324.
9. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154**:125–169.
10. **Dambaugh, T. R., C. A. Greenamoyer, E. D. Anton, J. J. O'Brian, G. J. Lindquester, and P. E. Pellett.** The nucleotide sequence of human herpesvirus 6B strain Z29 at a conserved locus encoding several replication genes reveals both conserved and novel protein sequences. (Genbank accession number L14772.). Submitted for publication.
11. **Dambaugh, T. R., J. J. O'Brian, E. D. Anton, C. A. Greenamoyer, G. J. Lindquester, and P. E. Pellett.** A 20.9 kb domain of human herpesvirus 6B (Z29) DNA colinear with human cytomegalovirus encodes homologs of genes from discontinuous loci in alpha- and gammaherpesviruses and contains a putative origin of HHV-6B DNA replication. (Genbank accession number 16947.). Submitted for publication.
12. **Dambaugh, T. R., and P. E. Pellett.** Unpublished data.
13. **Deb, S., and S. P. Deb.** 1989. Analysis of ori-s sequence of HSV-1: identification of one functional DNA binding domain. Nucleic Acids Res. **17**:2733–2752.
14. **Deb, S., and S. P. Deb.** 1991. A 269-amino-acid segment with a pseudo-leucine zipper and a helix-turn-helix motif codes for the sequence-specific DNA-binding domain of herpes simplex virus type 1 origin-binding protein. J. Virol. **65**:2829–2838.
15. **Dewhurst, S., B. Chandran, K. McIntyre, K. Schnabel, and C. B. Hall.** 1992. Phenotypic and genetic polymorphisms among human herpesvirus-6 isolates from North American infants. Virology **190**:490–493.
16. **Dewhurst, S., S. C. Dollard, P. E. Pellett, and T. R. Dambaugh.** 1993. Identification of a lytic-phase origin of DNA replication in human herpesvirus 6B strain Z29. J. Virol. **67**:7680–7683.
17. **Dewhurst, S., D. M. Krenitsky, and C. Dykes.** Unpublished data.
18. **Dewhurst, S., K. McIntyre, K. Schnabel, and C. B. Hall.** 1993. Human herpesvirus 6 (HHV-6) variant B accounts for the majority of symptomatic primary HHV-6 infections in a population of U.S. infants. J. Clin. Microbiol. **31**:416–418.
19. **Dinstein, R., D. Heffetz, Y. Ben-Neriah, and Y. Shaul.** 1992. c-abl has a sequence-specific enhancer binding activity. Cell **69**:751–757.
20. **Efstathiou, S., G. L. Lawrence, C. M. Brown, and B. G. Barrell.** 1992. Identification of homologues to the human cytomegalovirus US22 gene family in human herpesvirus 6. J. Gen. Virol. **73**:1661–1671.
21. **Elias, P., C. M. Gustafsson, and O. Hammarsten.** 1990. The origin binding protein of herpes simplex virus 1 binds cooperatively to the viral origin of replication ori_s. J. Biol. Chem. **265**:17167–17173.
22. **Elias, P., and I. R. Lehman.** 1988. Interaction of origin binding protein with an origin of replication of herpes simplex virus 1. Proc. Natl. Acad. Sci. USA **85**:2959–2963.
23. **Elias, P., M. E. O'Donnell, E. S. Mocarski, and I. R. Lehman.** 1986. A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **83**:6322–6326.
24. **Fixman, E. D., G. S. Hayward, and S. D. Hayward.** 1992. *trans*-acting requirements for replication of Epstein-Barr virus ori-Lyt. J. Virol. **66**:5030–5039.
25. **Gompels, U. A., A. L. Carss, N. Sun, and J. R. Arrand.** 1992. Infectivity determinants encoded in a conserved gene block of human herpesvirus-6. DNA Seq. **3**:25–39.
26. **Hammerschmidt, W., and B. Sugden.** 1988. Identification and characterization of *oriLyt*, a lytic origin of DNA replication of Epstein-Barr virus. Cell **55**:427–433.
27. **Hazuda, D. J., H. C. Perry, A. M. Naylor, and W. L. McClements.** 1991. Characterization of the herpes simplex virus origin binding protein interaction with ori_s. J. Biol. Chem. **266**:24621–24626.
28. **Inoue, N., and P. E. Pellett.** Unpublished data.
29. **Klupp, B. G., H. Kern, and T. C. Mettenleiter.** 1992. The virulence-determining genomic BamHI fragment 4 of pseudorabies virus contains genes corresponding to the U_L15 (partial). U_L18, U_L19, U_L20, and U_L21 genes of herpes simplex virus and a putative origin of replication. Virology **191**:900–908.
30. **Knowles, W. A., and S. D. Gardner.** 1988. High prevalence of antibody to human herpesvirus-6 and seroconversion associated with rash in two infants. Lancet **ii**:912–913.
31. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
33. **Lawrence, G. L., M. Chee, M. A. Craxton, U. A. Gompels, R. W. Honess, and B. G. Barrell.** 1990. Human herpesvirus 6 is closely related to human cytomegalovirus. J. Virol. **64**:287–299.
34. **Lindquester, G. J., N. Inoue, R. D. Allen, J. W. Castelli, F. R. Stamey, T. R. Dambaugh, J. J. O'Brian, R. M. Danovich, N. Frenkel, and P. E. Pellett.** Unpublished data.
35. **Lindquester, G. J., and P. E. Pellett.** 1991. Properties of the human herpesvirus 6 strain Z29 genome: G + C content, length, and presence of variable-length directly repeated terminal sequence elements. Virology **182**:102–110.
36. **Malik, A. K., R. Martinez, L. Muncy, E. P. Carmichael, and S. K. Weller.** 1992. Genetic analysis of the herpes simplex virus type 1 UL9 gene: isolation of a lacZ insertion mutant and expression in eukaryotic cells. Virology **190**:702–715.
37. **Martin, M. E. D., B. J. Thomson, R. W. Honess, M. A. Craxton, U. A. Gompels, M. Y. Liu, E. Littler, J. R. Arrand, I. Teo, and M. D. Jones.** 1991. The genome of human herpesvirus 6: maps of unit-length and concatemeric genomes for nine restriction endonucleases. J. Gen. Virol. **72**:157–168.
38. **Martinez, R., L. Shao, and S. K. Weller.** 1992. The conserved helicase motifs of the herpes simplex virus type 1 origin-binding protein UL9 are important for function. J. Virol. **66**:6735–6746.
39. **McGeoch, D. J., M. A. Dalrymple, A. Dolan, D. McNab, L. J. Perry, P. Taylor, and M. D. Challberg.** 1988. Structures of herpes simplex virus type 1 genes required for replication of virus DNA. J. Virol. **62**:444–453.
40. **Nakajima-Iijima, S., H. Hamada, P. Reddy, and T. Kakunaga.** 1985. Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. Proc. Natl. Acad. Sci. USA **82**:6133–6137.
41. **Neipel, F., K. Ellinger, and B. Fleckenstein.** 1991. The unique region of the human herpesvirus 6 genome is essentially collinear with the U_L segment of human cytomegalovirus. J. Gen. Virol. **72**:2293–2297.
42. **Olivo, P. D., N. J. Nelson, and M. D. Challberg.** 1988. Herpes simplex virus DNA replication: the UL9 gene encodes an origin-binding protein. Proc. Natl. Acad. Sci. USA **85**:5414–5418.
43. **Pari, G. S., M. A. Kacica, and D. G. Anders.** 1993. Open reading frames UL44, IRS1/TRS1, and UL36-38 are required for transient complementation of human cytomegalovirus *ori*Lyt-dependent

DNA synthesis. J. Virol. **67:**2575–2582.
44. **Pellett, P. E., G. J. Lindquester, P. Feorino, and C. Lopez.** 1990. Genomic heterogeneity of human herpesvirus 6 isolates. Adv. Exp. Med. Biol. **278:**9–18.
45. **Pruksananonda, P., C. B. Hall, R. A. Insel, K. McIntyre, P. E. Pellett, C. E. Long, K. C. Schnabel, P. H. Pincus, F. R. Stamey, T. R. Dambaugh, and J. A. Stewart.** 1992. Primary human herpesvirus 6 infection in young children. N. Engl. J. Med. **326:**1445–1450.
46. **Roizman, B., R. C. Desrosiers, B. Fleckenstein, C. Lopez, A. C. Minson, and M. J. Studdert.** 1992. The family Herpesviridae: an update. Arch. Virol. **123:**425–449.
47. **Sánchez-Martínez, D., and P. E. Pellett.** 1991. Expression of HSV-1 and HSV-2 glycoprotein G in insect cells by using a novel baculovirus expression vector. Virology **182:**229–238.
48. **Saxinger, C., H. Polesky, N. Eby, S. Grufferman, R. Murphy, G. Tegtmeir, V. Parekh, S. Memon, and C. Hung.** 1988. Antibody reactivity with HBLV (HHV-6) in U.S. populations. J. Virol. Methods **21:**199–208.
49. **Schirmer, E. C., L. S. Wyatt, K. Yamanishi, W. J. Rodriguez, and N. Frenkel.** 1991. Differentiation between two distinct classes of viruses now classified as human herpesvirus 6. Proc. Natl. Acad. Sci. USA **88:**5922–5926.
50. **Stamey, F. R., J. B. Black, K. Kite-Powell, T. R. Dambaugh, and P. E. Pellett.** Unpublished data.
51. **Stow, N. D., and A. J. Davison.** 1986. Identification of a varicella-zoster virus origin of DNA replication and its activation by herpes simplex type 1 gene products. J. Gen. Virol. **67:**1613–1623.
52. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin **1555.** College Station, Tex.
53. **Telford, E. A. R., M. S. Watson, K. McBride, and A. J. Davison.** 1992. The DNA sequence of equine herpesvirus-1. Virology **189:**304–316.
54. **Thomson, B. J., S. Efstathiou, and R. W. Honess.** 1991. Acquisition of the human adeno-associated virus type-2 *rep* gene by human herpesvirus type-6. Nature (London) **351:**78–80.
55. **Weir, H. M., J. M. Calder, and N. D. Stow.** 1989. Binding of the herpes simplex virus type 1 UL9 gene product to an origin of viral DNA replication. Nucleic Acids Res. **17:**1409–1425.
56. **Weller, S. K., A. Spadaro, J. E. Schaffer, A. W. Murray, A. M. Maxam, and P. A. Schaffer.** 1985. Cloning, sequencing, and functional analysis of ori_L, a herpes simplex virus type 1 origin of DNA synthesis. Mol. Cell. Biol. **5:**930–942.
57. **Wu, C. A., N. J. Nelson, D. J. McGeoch, and M. D. Challberg.** 1988. Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J. Virol. **62:**435–443.
58. **Wyatt, L. S., N. Balachandran, and N. Frenkel.** 1990. Variations in the replication and antigenic properties of human herpesvirus 6 strains. J. Infect. Dis. **162:**852–857.
59. **Yamanishi, K., T. Okuno, K. Shiraki, M. Takahashi, T. Kondo, Y. Asano, and T. Kurata.** 1988. Identification of human herpesvirus-6 as a casual agent for exanthem subitum. Lancet **i:**1065–1067.
60. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.