

Human herpesvirus-6 rep/U94 gene product has single-stranded DNA-binding activity

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The characterization is reported of the human herpesvirus-6B (HHV-6B) rep/U94 gene, which is a homologue of the adeno-associated virus type 2 rep. In this study, a monoclonal antibody was produced against HHV-6B REP (anti-REP mAb). Immunofluorescence staining using the anti-REP mAb showed that REP was localized to the nucleus in HHV-6-infected MT4 cells. It was first detected at 24 h post-infection (p.i.) and accumulated to higher levels by 72 h p.i. REP may be expressed only at very low levels in HHV-6-infected cells: even when the late protein glycoprotein H was detected in nearly 90% of HHV-6-infected cells, REP was detected in only a small percentage of them. Western blot analysis showed that the anti-REP mAb recognized a 56-kDa polypeptide in HHV-6B-infected MT4 cells. Furthermore, the REP protein was shown to bind single-stranded DNA.

Introduction

Human herpesvirus 6 (HHV-6) was first isolated from peripheral blood of patients with lymphoproliferative disorders (Salahuddin *et al.*, 1986). HHV-6 is now classified into two variants: HHV-6A and HHV-6B (Ablashi, 1993). HHV-6A has not been associated aetiologically with any disease, whereas HHV-6B is the aetiological agent of exanthem subitum (Yamanishi *et al.*, 1988).

Both HHV-6 variants, represented by HHV-6A strain U1102 (Gompels *et al.*, 1995) and HHV-6B strains HST (Isegawa *et al.*, 1999) and Z29 (Dominguez *et al.*, 1999), contain a linear, double-stranded DNA genome of approximately 161 kbp with 112 potential open reading frames (ORFs). Nucleotide sequencing has shown that HHV-6A and HHV-6B contain an ORF, U94, that encodes a 490-amino-acid protein homologous to Rep 78/68, a non-structural protein from the human parvovirus adeno-associated virus type 2 (AAV-2) (Thomson *et al.*, 1991; Gompels *et al.*, 1995; Isegawa *et al.*, 1999; Dominguez *et al.*, 1999). Interestingly, the AAV-2 rep gene homologue is unique to HHV-6 and is not present in other human herpesviruses, making the role of HHV-6 REP in the life-cycle of HHV-6 of particular interest. A rep homologue

has recently been identified in the genome of rat cytomegalovirus (Vink *et al.*, 2000).

AAV-2 Rep is known to possess several biological activities, including DNA binding and site- and strand-specific endonuclease, helicase and ATPase activities, all of which are required for AAV-2 DNA replication (Im & Muzyczka, 1990, 1992). A distinctive feature of AAV-2 is that the virus DNA integrates preferentially within a defined region of the cellular genome (Linden *et al.*, 1996). HHV-6 has also been reported to integrate into the human genome (Luppi *et al.*, 1993, 1994; Torelli *et al.*, 1995; Daibata *et al.*, 1998). Conservation between HHV-6 and AAV-2 may mean, therefore, that HHV-6 REP possesses a similar range of functions advantageous to the survival of HHV-6 within the host. In support of this idea, HHV-6 REP has been shown to complement replication of a rep-deficient AAV-2 genome (Thomson *et al.*, 1994), suggesting that they might have similar biological functions.

Recently, we reported the results of immunohistochemistry experiments using a polyclonal antibody that showed that HHV-6 REP was localized to the nucleus of HHV-6-infected cells and that the protein was expressed at the late stage of virus infection (Mori *et al.*, 2000). However, we could not detect the REP protein in HHV-6-infected T cells by either Western blotting or immunoprecipitation using the polyclonal antibody. This observation prompted us to make monoclonal antibodies (mAbs) against REP. In this study, we have isolated

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mAbs against REP and detected the protein in HHV-6-infected T cells by Western blotting. Furthermore, we report here that REP possesses single-stranded (ss) DNA-binding activity.

Methods

Cells and viruses. Umbilical cord blood mononuclear cells (CBMCs) were separated on a Ficoll–Conray gradient, cultured in RPMI 1640 containing 10% foetal calf serum and stimulated with phytohaemagglutinin (5 µg/ml) for 2 or 3 days. In order to prepare virus stocks, HHV-6 strains HST and U1102 were propagated in stimulated CBMCs as described previously (Mori *et al.*, 1998). When more than 80% of cells exhibited a cytopathic effect, the culture of infected cells was frozen and thawed twice. After centrifugation at 1500 g for 10 min, the supernatant was stored at –80 °C as cell-free virus stock. A recombinant vaccinia virus (vTF7) that expresses T7 RNA polymerase was a kind gift from Bernard Moss (National Institutes of Health, Bethesda, MD, USA) and was grown in Vero cells.

Expression of the rep gene in *E. coli*. A DNA fragment comprising the entire amino acid coding region of HHV-6B rep was amplified by PCR and cloned, in-frame, into the pMALTM-c2 bacterial expression vector (New England Biolabs) at the *Bam*HI and *Sal*I sites. This vector also contained the coding region for maltose-binding protein (MBP) and the resultant fusion protein (MBP–REP) was expressed in *E. coli* DH5α.

Preparation of REP by using recombinant baculovirus. A DNA fragment comprising the full-length rep gene ORF was amplified by PCR and inserted into the *Bam*HI and *Hind*III sites of the pFastBac donor plasmid (Gibco BRL). The nucleotide sequences of the primers used for the PCR were: sense, 5' ggatccCCACCATGTTTTCCATAATAAA-TCCAAGT 3'; antisense, 5' agacttGTAAAATTTTTGGAACCGTG-TAGTC 3' (lower-case letters indicate additional restriction sites). The pFastBac-recombinant virus containing REP was prepared in accordance with the manufacturer's protocol (Bac to BAC Baculovirus expression systems; Gibco BRL).

Establishment of mAbs. BALB/c mice were immunized with AcGST–REP, a purified fusion of glutathione S-transferase (GST)–REP–*myc* (Mori *et al.*, 2000), and boosted with MBP–REPNI, the purified fusion of MBP–REP (Mori *et al.*, 2000), as described previously (Okuno *et al.*, 1990). The first immunization was carried out with 100 µg GST–REP–*myc* in complete Freund's adjuvant and followed by three boosters with 100 µg MBP–REPNI, 3–4 weeks apart, in incomplete Freund's adjuvant.

Hybridomas were established by fusing splenocytes from the hyperimmune mice with the non-producing myeloma cell line Sp2/0-Ag14. After selection in medium containing hypoxanthine/aminopterin/thymidine, cells secreting mAbs were screened by indirect immunofluorescence assays (IFA). Clones secreting antibodies reactive with HHV-6B- (HST strain) infected MT4 cells and baculovirus–REP- (Bac–REP) infected Sf9 cells were expanded and cloned by limiting dilutions. Ascites fluids with high antibody titres were then accumulated by injecting cloned hybrid cells intraperitoneally into mice treated with Pristane (Sigma).

Immunohistochemical analysis of HST-infected MT4 cells. In order to confirm the REP expression patterns, immunohistochemical analysis was carried out using the anti-REP mAb as described previously (Mori *et al.*, 2000). HST-infected MT4 cells were collected at 12, 24, 48 and 72 h post-infection (p.i.). The cells were fixed in cold acetone and

incubated at 37 °C for 1 h with primary antibodies: the anti-REP mAb, OHV-2, which recognizes a nuclear protein expressed in the early stage, or OHV-3, which recognizes HHV-6B glycoprotein H (gH) expressed in the late stage. After washing with PBS for 10 min, fluorescein-conjugated goat antibodies against mouse IgG were added with saturated 4',6'-diamidino-2-phenylindole (DAPI) at a 1:100 dilution. The cells were incubated for 20 min. After washing as above, signals were detected by confocal microscopy.

Western blot analysis. Western blot analysis was carried out as described previously (Mori *et al.*, 2000). Cell lysates were prepared from mock- and HST-infected MT4 cells, mock- and Bac–REP-infected Sf9 cells or mock- and vaccinia virus-infected 293T cells transfected with pcDNArepB (Mori *et al.*, 2000) at 72 h p.i. Samples were subjected to 8% SDS–PAGE, transferred to PVDF membranes (Bio-Rad) and reacted with the primary antibodies. Reactive bands were visualized using horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence detection reagents (ECL; Amersham Pharmacia).

Preparation of nuclear extracts. Nuclear extracts were prepared as described previously (Ni *et al.*, 1994). After harvesting the cells by centrifugation, 5×10^6 SupT1 cells were washed once by suspension in buffer A (20 mM HEPES–KOH, pH 7.5, 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM DTT) containing 10% sucrose. The cells were then suspended in 500 µl buffer A and incubated on ice for 30 min. They were then lysed by sonication. Nuclei were collected by centrifugation at 2000 g for 10 min and resuspended in 100 µl buffer B (50 mM HEPES–KOH, pH 7.5, 10% sucrose, 0.4 mM EDTA, 0.3 mM PMSF, 2 µg/ml leupeptin and 1 µg/ml pepstatin). The suspension was adjusted to 1 M NaCl and incubated at 4 °C for 1 h and then spun at 100 000 g for 1 h at 4 °C and frozen at –70 °C.

Preparation of cytoplasmic and nuclear extracts from baculovirus-infected Sf9 cells. Sf9 cells (2×10^7) were grown in Grace's insect cell culture medium (Gibco BRL). The cells were infected with recombinant baculovirus (m.o.i. of 5–10) and incubated at 27 °C for 3 days. Cytoplasmic and nuclear extracts were prepared as described previously (Ni *et al.*, 1994). Briefly, after harvesting by centrifugation, the cells were washed once with buffer A supplemented with 10% sucrose. The cells were then suspended in 1 ml buffer A, incubated on ice for 30 min, lysed by sonication and spun at 2000 g for 10 min. The supernatant was collected as the cytoplasmic fraction and the pellet containing the nuclei was resuspended in 200 µl buffer B. The suspension was adjusted to 1 M NaCl and incubated at 4 °C for 1 h and then spun at 100 000 g for 1 h at 4 °C. Five µl of each fraction was subjected to 8% SDS–PAGE, immunoblotted onto PVDF membranes and probed with the anti-REP mAb.

REP DNA-binding assay. The affinity between REP and DNA was examined by monitoring the elution profile of REP by DNA-affinity column chromatography (Tanaka *et al.*, 1999). Sixty µg each of the fusion protein MBP–REP and MBP in binding buffer (100 mM NaCl, 10 mM Tris–HCl, pH 7.4, 25 mM KCl, 0.05% Tween 20, 1 mM PMSF and 0.5 mM EDTA) was applied to 500 µl unmodified cellulose or ssDNA–cellulose (Pharmacia Biotech) in polyprep columns (Bio-Rad). Each column was washed with 250 ml binding buffer and the materials were eluted step-wise with 500 µl volumes of 0.1, 0.2, 0.3, 0.4, 0.6 and 1 M NaCl in binding buffer. Twenty µl each of the input, flow-through, first wash and fractions of each salt concentration were subjected to Western blot analysis with the anti-MBP polyclonal antibody.

In order to determine whether a cellular factor is involved in the binding of REP to ssDNA, nuclear extracts from 5×10^6 SupT cells were mixed with 30 µg of each protein and applied to unmodified cellulose or

ssDNA–cellulose columns. The binding assay was carried out as described above.

■ **Probes.** The DNA fragments used in the mobility-shift assay were prepared by synthesis of oligonucleotides. The DNA fragments were 5'-end labelled with [γ - 32 P]ATP and T4 polynucleotide kinase. The Δ ITR probes (Chiorini *et al.*, 1994) for double-stranded (ds) DNA binding were made by 5'-end labelling either oligomer and annealing with the unlabelled complementary oligonucleotide. Δ ITR oligonucleotides were produced synthetically as described previously (Chiorini *et al.*, 1994). The probe sequences were: AD' (Δ ITR sense), 5' GATCAGTGATGGA GTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCC 3'; A'D (Δ ITR antisense), 5' CTAGGGCTCAGTGAGCGAGCGA-GCGCGCAGAGAGGGAGTGGCCAATCCATCACT 3'; and 68-mer oligonucleotide, 5' CTCGGTACCTCGAGTGAAGCTTGA(N₂₅) GGGAAATTCGGATCCGCGTAAC 3'.

■ **Mobility-shift assays.** DNA–protein complexes were detected by monitoring the electrophoretic mobility of 32 P-labelled probes on a non-denaturing gel. For ssDNA binding, 32 P-end-labelled 68-mer oligonucleotide and Δ ITR AD' were used as probes. The reaction was performed in buffer (30 mM HEPES–KOH, pH 7.5, 7 mM MgCl₂, 4 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, 5% glycerol) with either MBP–REP or MBP and DNA for 30 min at room temperature. Experiments involving competition between the radiolabelled DNA substrates were performed as described in the figure legends. The samples were loaded on composite gels (2.5% acrylamide and 0.6% agarose) and subjected to electrophoresis in TBE buffer.

For dsDNA binding, radiolabelled probes were incubated with protein at 30 °C for 15 min in 25 μ l. The reaction mixture contained 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 4% glycerol and 0.5 μ g poly(dI.dC). MBP–Rep68 Δ (Chiorini *et al.*, 1994), used as a positive control for dsDNA binding, was kindly provided by Robert M. Kotin (Molecular Hematology Branch, National Heart, Lung and Blood Institute, Bethesda, MD, USA).

Results

Expression of REP in Bac–REP-infected Sf9 cells

In order to characterize the activity of the mAb against REP (anti-REP mAb), an IFA and immunoblotting were performed using the mAb. As shown in Fig. 1(b), REP was detected by IFA at very high levels in Bac–REP-infected Sf9 cells. With Western blotting, REP was detected in both the nucleus and cytoplasm at high levels (Fig. 1f).

Expression of REP in MT4 cells infected with strain HST

IFAs were performed using the anti-REP mAb to confirm the expression of REP in HHV-6-infected cells. REP was expressed in HHV-6-infected MT4 cells. It was first detected at 24 h p.i. (data not shown) and accumulated to higher levels by 72 h p.i. (Fig. 2c, e). In addition, no specific labelling was found in mock-infected cells (Fig. 2d). At a late stage of infection (72 h p.i.), although the late protein gH was detected in nearly 90% of HHV-6-infected cells (Fig. 2b), REP was detected in only a small percentage of them (Fig. 2c). In order to examine the localization of REP, HHV-6B-infected MT4 cells were

stained with anti-REP mAb and DAPI on the same slide. As shown in Fig. 2(e–g), REP staining overlapped with DAPI staining, indicating that REP was located in the nucleus.

In vitro translation of the HHV-6 rep ORF

For *in vitro* translation, a plasmid was constructed by cloning amplified PCR products into pcDNA3.1 under the control of the T7 promoter. *In vitro* translation of pcDNArepA (HHV-6A rep gene) and pcDNArepB (HHV-6B rep gene) produced a prominent 56-kDa protein band (Fig. 3a, lanes 2 and 3). The observed size of the *in vitro*-translated REP protein (56 kDa) corresponded well to the calculated size of 56 kDa based on the predicted amino acid sequence.

REP mAb recognizes the REP protein in Bac–REP-infected Sf9 cells and HHV-6-infected MT4 cells by Western blotting

Previously, we could not detect REP by Western blotting with our polyclonal antibody. In order to determine the molecular mass of REP, we performed Western blotting using the anti-REP mAb. Fig. 3(c) shows that the anti-REP mAb recognized a 56-kDa polypeptide in Bac–REP-infected Sf9 cells, HST-infected MT4 cells and pcDNA repB-transfected 293T cells (Fig. 3c, lanes 2, 4 and 6). No specific band was obtained from mock-infected Sf9 cells, mock-infected MT4 cells or pcDNA-transfected 293T cells, the negative controls (Fig. 3c, lanes 1, 3 and 5).

We also analysed the proteins from the *in vitro* translation (Fig. 3c, lane 8) and found that the molecular mass was similar to that seen in HST-infected cells. This suggests that there was no major post-translational modification. In addition, we could not detect HHV-6A REP in HHV-6A-infected CBMCs (data not shown) or in an *in vitro* translation of HHV-6A REP (Fig. 3b, lane 2) using the anti-REP mAb. As shown in Fig. 3(a, b), the anti-REP mAb reacted with *in vitro* translation products of pcDNArepB (HHV-6B) (Fig. 3a, b; lanes 3) but not pcDNArepA (HHV-6A) (Fig. 3a, b; lanes 2). Furthermore, the mAb reacted with the amino terminus of REP expressed from pcDNA3.1 (Fig. 3a, b; lanes 4) but not the carboxy terminus (Fig. 3a, b; lanes 5). Therefore, the mAb reacted with HHV-6B REP specifically and recognized the amino terminus of REP. The major products corresponding to the REP amino terminus (Fig. 3a, b; lanes 4) and full-length REP (Fig. 3c, lanes 6 and 8) were seen as two bands; these protein mobilities correspond to those predicted from the use of two different start codons, as reported by Rapp *et al.* (2000).

REP has ssDNA-binding activity

DNA–cellulose chromatography was performed in order to determine whether HHV-6 REP could bind ssDNA. The MBP–REP fusion protein or MBP protein was applied to

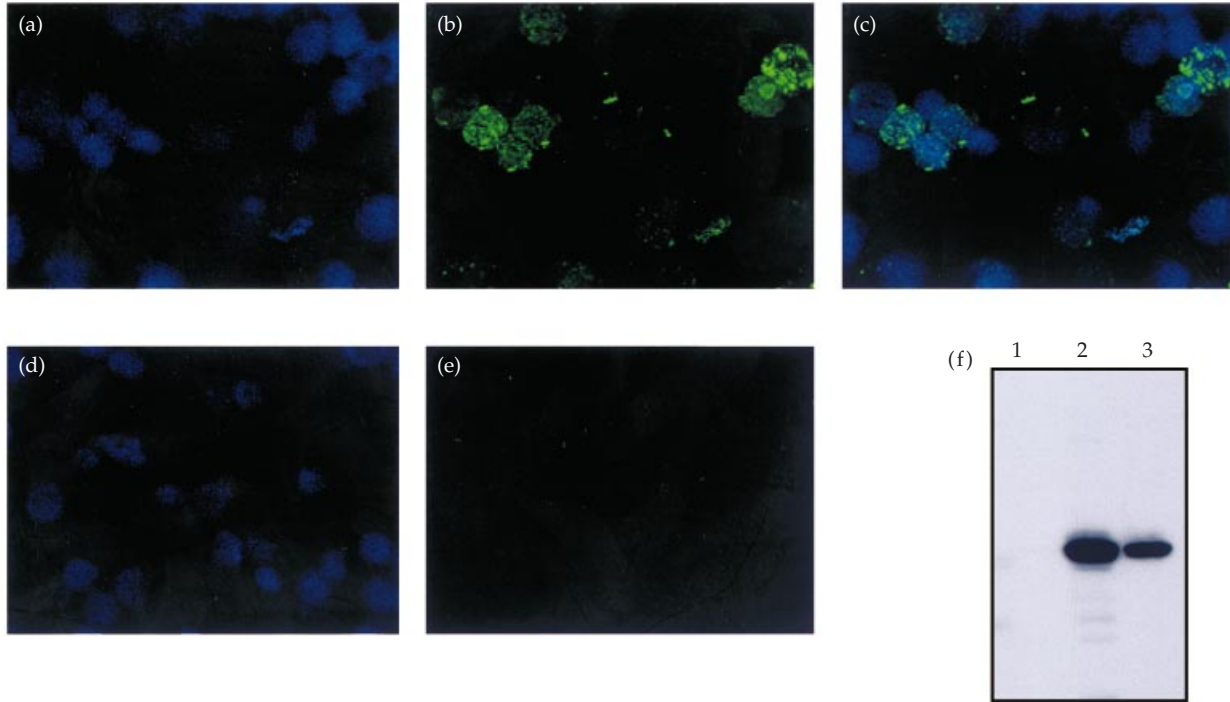


Fig. 1. Cellular localization of HHV-6B REP expression in Sf9 cells. (a)–(e) Sf9 cells infected with recombinant baculovirus for 72 h were stained with DAPI (a) and anti-REP mAb (b). Panel (c) shows (a) and (b) overlaid. Mock-infected Sf9 cells were stained with DAPI (d) and anti-REP mAb (e). Specific immunofluorescence was observed under a confocal laser scanning microscope (Nikon TE300, Bio-Rad Radiance2100) at 40 × magnification. (f) Western blot analysis of REP expression in Sf9 cells. Lanes: 1, mock-infected Sf9 cells; 2–3, cytoplasmic fraction (2) and nuclear extract (3) of Sf9 cells infected with recombinant baculovirus for 72 h.

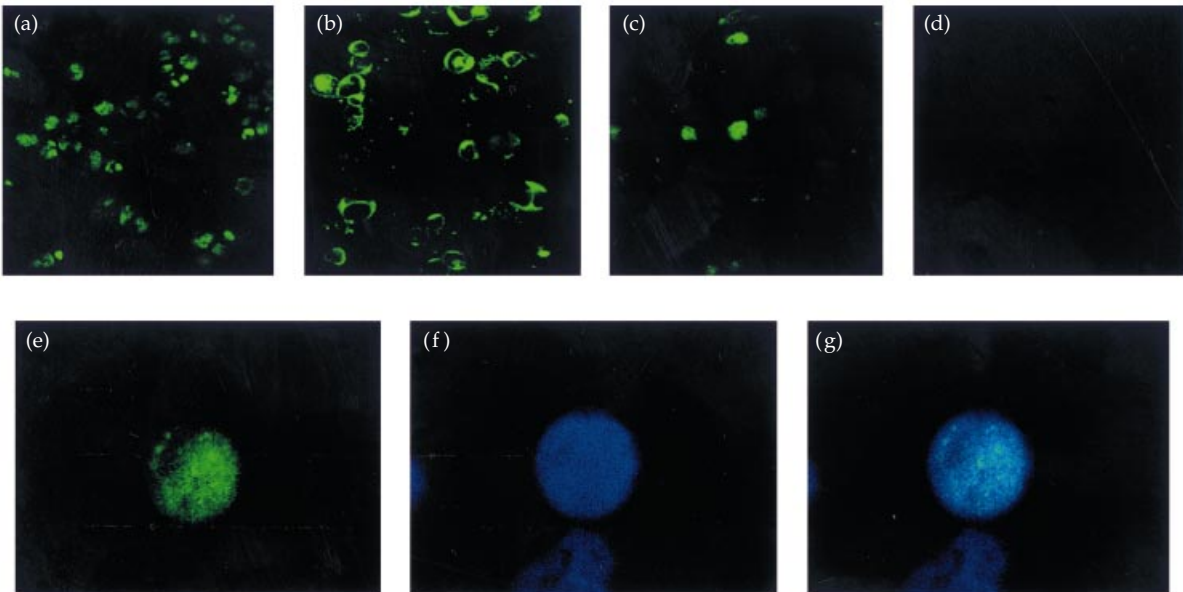


Fig. 2. Cellular localization of HHV-6B REP in MT4 cells infected with HST for 72 h. (a)–(d) Confocal microscopy (Carl Zeiss) performed on HHV-6-infected MT4 cells stained with (a) OHV-2, a mAb against HHV-6 nuclear antigen, which is expressed in the early stage, (b) OHV-3, a mAb against the HHV-6 gH antigen, which is expressed in the late stage, and (c) the anti-REP mAb. (d) Mock-infected MT4 cells stained with anti-REP mAb. Magnification, × 40. (e)–(g) MT4 cells infected with HST for 72 h were stained with anti-REP mAb (e) and DAPI (f). Panel (g) shows (e) and (f) overlaid. Specific immunofluorescence was observed under a confocal laser scanning microscope (Nikon TE300, Bio-Rad Radiance2100). Magnification, × 60.

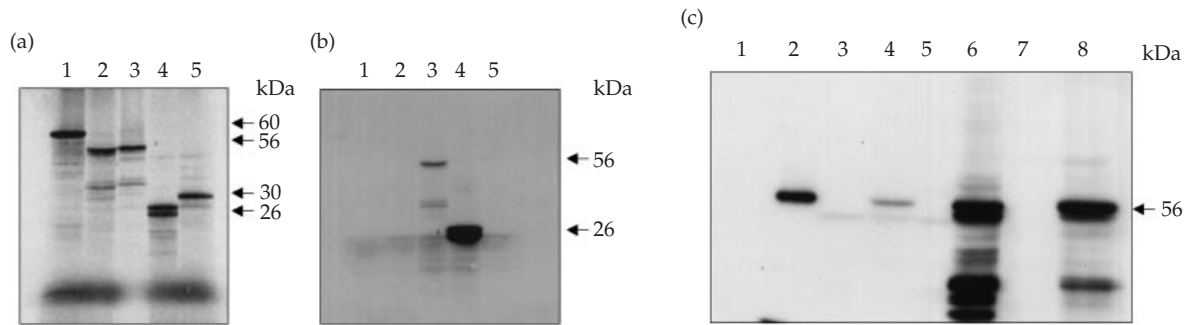


Fig. 3. HHV-6B-specific reactivity of anti-REP mAb. (a)–(b) *In vitro* translation products of the rep gene were boiled in SDS sample buffer and subjected to 12% SDS-PAGE and immunoblotting with the anti-REP mAb. *In vitro* translation products labelled with [³⁵S]methionine are shown in (a) and Western blot analysis of the *in vitro* translation products with the anti-REP mAb is shown in (b). Lanes: 1, luciferase gene; 2, pcDNArepA; 3, pcDNArepB; 4, fragment encoding the amino terminus of REP in pcDNA3.1; 5, fragment encoding the carboxy terminus of REP in pcDNA 3.1. (c) Western blot analysis of REP protein. Cells were collected by centrifugation, washed, boiled in SDS sample buffer and subjected to SDS-PAGE and Western blotting with the anti-REP mAb. Lanes: 1, mock-infected Sf9 cells; 2, Bac-REP-infected Sf9 cells; 3, mock-infected MT4 cells; 4, HST-infected MT4 cells; 5, 293T cells infected with pTF7 recombinant vaccinia virus and then transfected with pcDNA3.1; 6, 293T cells infected with pTF7 and then transfected with pcDNArepB; 7, *in vitro* translation product of the luciferase gene; 8, *in vitro* translation product of pcDNArepB. *In vitro* transcription and translation from the plasmids was carried out using the TNT T7-coupled reticulocyte lysate system.

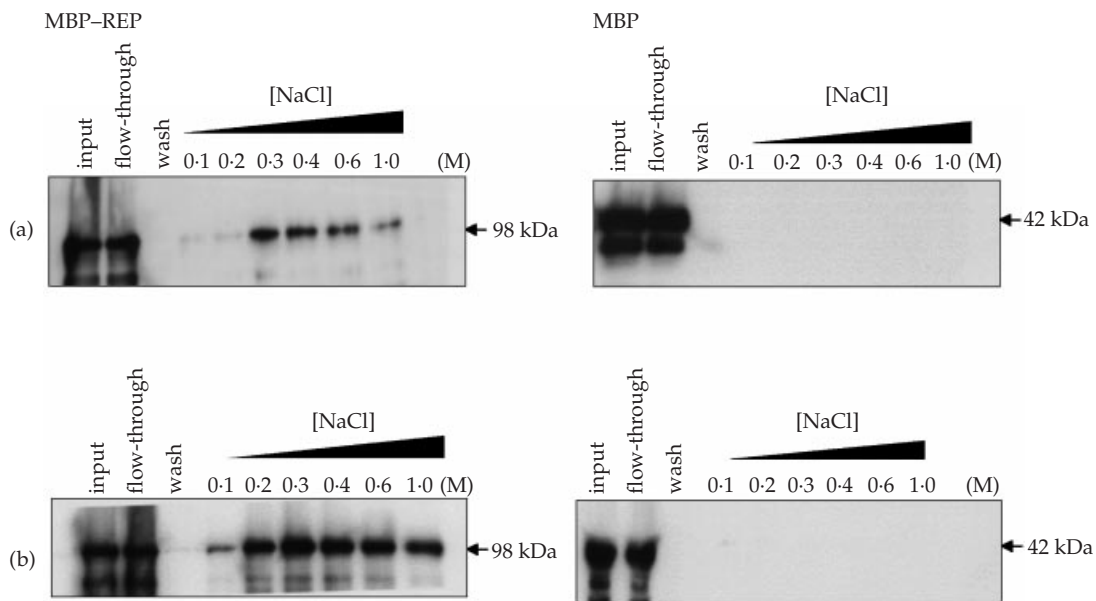


Fig. 4. REP ssDNA-binding activity. (a) Either the MBP-REP fusion protein or the MBP protein was applied to ssDNA-cellulose in a column. The columns were washed extensively and eluted step-wise with increasing concentrations of NaCl in binding buffer. Twenty μ l of fractions from each NaCl concentration were resolved by 8% SDS-PAGE and subjected to Western blotting. (b) Nuclear extracts of 5×10^6 SupT cells were mixed with 30 μ g of each protein and applied to ssDNA-cellulose columns. The sizes of the protein markers are shown on the right.

ssDNA-cellulose, as described in Methods. As shown in Fig. 4(a), the ssDNA-cellulose column retained MBP-REP, but did not retain MBP. To examine this more precisely, the ssDNA-binding activity of HHV-6 REP was also tested by gel-shift experiments using the 5'-end-labelled ssDNA oligonucleotides 68-mer nucleotide and Δ ITR AD'.

MBP-REP-ssDNA complexes in the mixture were identified by electrophoresis on composite gels (Fig. 5a, b; lanes 2),

but MBP-ssDNA complexes were not identified (Fig. 5a, b; lanes 3). Furthermore, the specificity of the ssDNA-binding activity of HHV-6 REP was addressed by incubating MBP-REP and ³²P-labelled probes with two different competitors. In the presence of either competitor, the MBP-REP-ssDNA complexes disappeared (Fig. 5a, b; lanes 4 and 5). These results demonstrate that HHV-6 REP possesses sequence-independent ssDNA-binding activity.

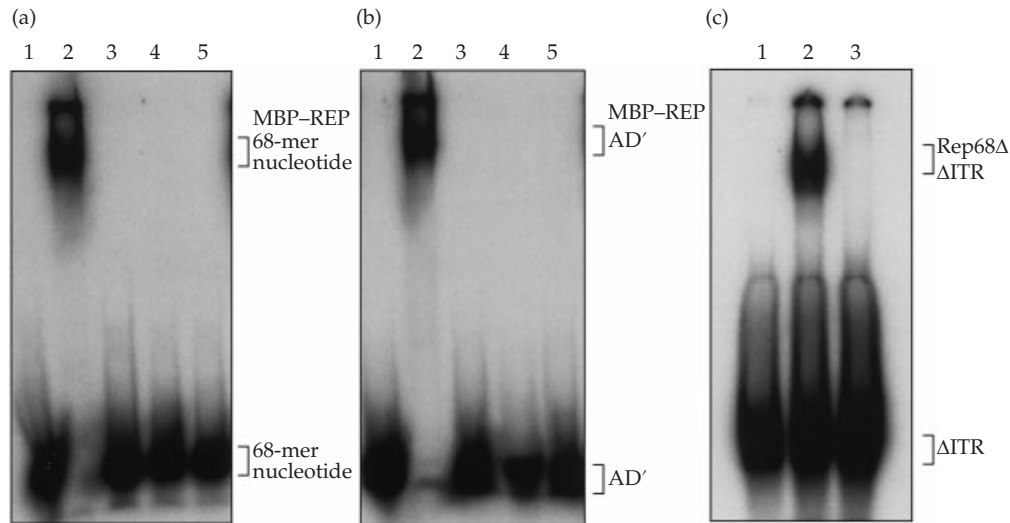


Fig. 5. Autoradiographs of mobility-shift assays. MBP-REP fusion protein and MBP were incubated with end-labelled ssDNA and applied to a composite gel (2.5% acrylamide and 0.6% agarose) in TBE buffer. The conditions for the assay are described in Methods. Each reaction mixture contained 2–4 fmol labelled probe (10000 c.p.m.) and 1 µg MBP-REP or MBP. (a) Binding activity of MBP-REP with 68-mer nucleotide. The positions of the free probe and the MBP-REP complex are indicated. Lanes: 1, 68-mer probe only; 2, 1 µg MBP-REP; 3, 1 µg MBP; 4 and 5, ³²P-labelled 68-mer nucleotide and MBP-REP incubated with 20-fold excess of unlabelled 68-mer nucleotide (lane 4) or ΔITR AD' (lane 5) competitor. (b) Binding activity of MBP-REP with ΔITR AD'. The positions of the free probe and the MBP-REP complex are indicated. Lanes: 1, AD' probe only; 2, 1 µg MBP-REP; 3, 1 µg MBP; 4 and 5, ³²P-labelled AD' and MBP-REP incubated with 20-fold excess of unlabelled 68-mer nucleotide (lane 4) or AD' competitor (lane 5). (c) Rep 68Δ (lane 2) and MBP-REP (lane 3) were incubated with ³²P-labelled ΔITR dsDNA. Lane 1 contains probe only.

In order to determine whether the REP protein bound ssDNA with other cellular proteins, nuclear extracts from SupT1 were mixed with either the MBP-REP fusion protein or MBP. The mixtures were applied to ssDNA columns and then eluted. Individual fractions were analysed for the presence of REP by immunoblotting. REP was detected in fractions eluted with higher NaCl concentrations when the SupT1 nuclear extracts were included than when REP was applied alone as a fusion protein (Fig. 4b). In contrast, MBP protein was not detected in the eluates, suggesting that the binding observed with MBP-REP was not caused by the MBP protein expressed in *E. coli*.

The dsDNA-binding activity of HHV-6 REP was also tested by using DNA-cellulose chromatography with the same methods as the ssDNA column. The dsDNA-cellulose column did not retain MBP-REP, indicating that HHV-6 REP could not bind dsDNA non-specifically (data not shown). As AAV-2 Rep has been reported to bind specifically to dsDNA, we investigated whether HHV-6 REP would also bind to specific dsDNA sequences. To examine this, a mobility-shift assay was performed by using ³²P-labelled ΔITR, a specific sequence for AAV-2 Rep binding. MBP-Rep68Δ, the positive control, formed a stable complex with ds ΔITR (Fig. 5c, lane 2), but MBP-REP did not form a complex (Fig. 5c, lane 3). Even when the amount of MBP-REP protein was increased (maximum 5 µg), MBP-REP-ΔITR complexes were not found (data not shown). Although we also examined whether

MBP-REP could bind HHV-6 DNA fragments by using random pSTY clones (Isegawa *et al.*, 1999), MBP-REP did not bind the HHV-6 DNA fragments that we tested (data not shown), indicating that HHV-6 REP does not have dsDNA-binding characteristics similar to those of its counterpart from AAV-2.

Discussion

HHV-6 ORF U94 encodes a protein that is homologous to the AAV-2 rep gene product and is unique among human herpesviruses (Thomson *et al.*, 1991). However, although the function of the AAV-2 rep gene is well-documented (Kellermann & Ferenci, 1982; Labow *et al.*, 1986, 1987; Beaton *et al.*, 1989; Heilbronn *et al.*, 1990; Im & Muzyczka, 1990; Antoni *et al.*, 1991; Hermonat, 1994a, b; Hermonat *et al.*, 1996, 1997, 1998; Kyostio *et al.*, 1995; Lee & Young, 1998), the function of the HHV-6 rep gene remains unknown. Recently, it has been reported that overexpression of the HHV-6 U94 product in T cells represses HHV-6 replication and that U94 might be involved in the latency of HHV-6 (Rotola *et al.*, 1998).

In our previous study, we initially detected the HHV-6B REP protein in the nucleus at 24 h p.i. and found that it accumulated to high levels, mainly in the nucleus and, to a lesser extent, in the cytoplasm, within 72 h. However, an anti-REP polyclonal antibody did not detect the REP protein in the presence of phosphonoformic acid or cycloheximide and

actinomycin D at any time (Mori *et al.*, 2000). To allow further investigation, for this study, we produced an anti-REP mAb. Using this mAb, we detected the REP protein in the nucleus and cytoplasm of HST-infected MT4 cells 24 h p.i. by IFA (data not shown). However, REP could be detected in few cells, even when gH, which is expressed at the late stage of virus infection, was detected in almost all cells, indicating that REP might be expressed only at very low levels in HST-infected cells. Rapp *et al.* (2000) also reported that the U94 transcript is expressed at low levels and that its expression is tightly regulated in HHV-6-infected cells. They also suggested that the U94 protein might be required only in small amounts during infection.

We also investigated the pattern of REP expression in recombinant baculovirus-infected cells using our anti-REP mAb. By Western blotting and IFA, we detected REP expression in the nucleus and cytoplasm at high levels. These results may indicate that the titre of the anti-REP mAb is high. In contrast, we did not detect HHV-6A REP as an *in vitro* translation product or in HHV-6A (U1102)-infected CBMCs with the anti-REP mAb. Therefore, the anti-REP mAb seemed to recognize only HHV-6B REP. In addition, the mAb reacted with the amino terminus of the REP protein and there are six amino acid differences between HHV-6A and HHV-6B in the amino terminus of REP; one of them could therefore be an epitope of the mAb.

In an *in vitro* translation and in pcDNA-transfected cells, the major products corresponding to the amino terminus of REP and full-length REP were detected as two bands. These protein mobilities correspond to those predicted from the use of two different start codons, as reported by Rapp *et al.* (2000). However, only one band, of 56 kDa, was found in HHV-6B-infected MT4 cells and baculovirus-infected Sf9 cells, suggesting that the first start codon might be used for translation mainly in virus-infected cells.

In this study, by using two different methods, we report that a bacterially expressed MBP-REP fusion protein possesses ssDNA-binding activity and that, when MBP-REP fusion protein was mixed with nuclear extracts of SupT1 cells, the ssDNA-binding capacity increased. Although our results from the MBP-REP fusion protein-binding assay indicate that REP can bind ssDNA weakly in the absence of other proteins, the increased binding in the presence of the SupT1 nuclear factors supports the idea that REP may interact with other cellular proteins that themselves bind DNA, and that these interactions result in the strong and tight binding of ssDNA.

It has been reported that AAV Rep 68 and Rep 78 directly bind the transcriptional coactivator PC4, an ssDNA-binding protein (Weger *et al.*, 1999). PC4 also interacts with components of the general transcription machinery, namely TFIIA or the TATA box-binding protein (TBP), in a manner that is dependent upon the presence of TFIIA (Ge & Roeder, 1994). It is believed that PC4 functions early, during formation of the TFIIA-TFIID-promoter (DA) complex (Kaiser *et al.*, 1995), and

that its function is dependent both on TBP-associated factors and on TFIIF. Previously, we reported that HHV-6 REP could bind TBP *in vitro* and *in vivo* (Mori *et al.*, 2000). Taken together, the published results and those concerning ssDNA binding from this study indicate that HHV-6 REP may play a role in DA complex formation and that its function is in virus gene regulation.

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