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Virology 346 (2006) 402 – 414
VIROLOGY
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Human herpesvirus 6 (HHV-6) U94/REP protein inhibits betaherpesvirus replication

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Received 11 August 2005; returned to author for revision 9 November 2005; accepted 15 November 2005
Available online 20 December 2005

Abstract

Human herpesvirus 6 (HHV-6) is the only human herpesvirus encoding U94/rep, homologue to the parvovirus non-structural gene rep68/78. Results to date suggest that HHV-6 U94/rep might regulate viral gene expression and have a role in viral latency. To determine the effect of U94/REP upon viral replication, the protein was produced. The purified U94/REP retained the characteristic immunological features. It was internalized and localized in the nucleus of human cells, showing marked inhibitory activity on the replication of HHV-6 (both variants A and B). The effect of U94/REP was dose-dependent and sensitive to treatment with single-stranded but not double-stranded DNA. U94/REP inhibited the replication of other betaherpesviruses, HHV-7 and human cytomegalovirus, but had no effect on herpes simplex virus. These results confirm the action of U94/rep latency gene in the regulation of HHV-6 replication with implications for co-reactivations and latency of human betaherpesviruses.

Keywords: HHV-6; U94/rep gene; Betaherpesvirus inhibition

Introduction

Human herpesvirus 6 (HHV-6) is a member of the betaherpesvirus subfamily, it is closely related to human herpesvirus 7 (HHV-7) and more distantly to human cytomegalovirus (HCMV; Dominguez et al., 1999; Gompels et al., 1995; Salahuddin et al., 1986). Primary infection occurs in the early childhood and causes exanthem subitum and febrile illness (Hall et al., 1994; Yamanishi et al., 1988). Thereafter, HHV-6 establishes a latent infection, where it is tightly controlled, but can reactivate during immunosuppression, establishing severe diseases in immunodeficient individuals including transplant and HIV/AIDS patients (Carrigan and Knox, 1994; Drobyski et al., 1993a; Gompels, 2004; Lusso and Gallo, 1995). HHV-6 has been associated to thrombotic microangiopathy (Matsuda et al., 1999), encephalitis and meningo-encephalitis (Ishiguro et al., 1990), infectious mononucleosis (Steeper et al., 1990), persistent lymphadenopathy (Niederman et al., 1988), autoimmune disorders (Krueger et al., 1991), AIDS dementia (Knox and Carrigan, 1995; Lusso and Gallo, 1995) and to complications following solid organ and bone marrow transplantation, such as pneumonitis and graft rejection (Carrigan and Knox, 1994; Drobyski et al., 1993b; Ljungman, 2002; Razonable and Paya, 2002), as well as fatal encephalitis in bone marrow and stem-cell-transplanted patients (Rapaport et al., 2002; Zerr et al., 2002). Recent studies also point to more severe pathology, 'CMV disease' in betaherpesvirus co-reactivations in solid organ transplantation (Emery, 2001; Lautenschlager et al., 2002) as well as a possible link to multiple sclerosis (Cermelli et al., 2003; Chapenko et

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doi:10.1016/j.virol.2005.11.018

al., 2003; Goodman et al., 2003; Rotola et al., 2004; Tejada-Simon et al., 2002). Thus, factors regulating latent infection have important implications for HHV-6-associated pathology.

HHV-6 strains are divided in two variants: HHV-6A and HHV-6B, which share a substantial degree of homology, but might differ for cell tropism and pathological implications (Campadelli-Fiume et al., 1999; Clark, 2000; Gompels, 2004). HHV-6 DNA shows a typical betaherpesvirus genomic organization and encodes several genes that are conserved in all human herpesviruses (Gompels et al., 1995). A unique genomic characteristic of HHV-6 consists in the presence of ORF U94/rep, a gene encoded by both variants but absent in all other human herpesviruses, even in the closer related betaherpesviruses, HHV-7 and HCMV. Several studies suggest that U94/rep may have features of an HHV-6 specific 'latency' gene (Rotola et al., 1998; Turner et al., 2002; Yoshikawa et al., 2003). ORF U94/rep encodes a 490-amino-acid protein, highly conserved between HHV-6 A and B variants (Rapp et al., 2000). Interestingly, it is homologous to rep68/78, encoded by the human parvovirus adeno-associated virus type 2 (AAV-2), a DNA virus unrelated to HHV-6 (Thomson et al., 1991). The presence of U94/rep is unique among human herpesviruses, and only rat cytomegalovirus (RCMV) encodes a rep homologue, r127 rep (Vink et al., 2000), suggesting either a common betaherpesvirus ancestor or independent acquisitions as herpesviruses can act as helper virus for AAV.

AAV-2 rep gene product (REP) is a non-structural protein involved in several aspects of AAV-2 regulation of gene expression, including DNA binding, site- and strand-specific endonuclease, helicase and ATPase activities (Im and Muzyczka, 1990, 1992). The presence of an intact rep gene is necessary for the integration of AAV DNA within defined regions of the cellular genome (Linden et al., 1996a, 1996b). In addition, AAV-2 REP inhibits transcription from HIV-1 long terminal repeat (LTR) promoter in fibroblasts and T-cell lines (Oelze et al., 1994) and represses the expression of cellular oncogenes (Chiorini et al., 1996; Surosky et al., 1997).

HHV-6 U94/rep gene product shares 24% identity with AAV-2 REP at the amino-acid level, suggesting that it may possess a similar range of functions in viral replication and survival within the infected host. This hypothesis is confirmed by the observation that HHV-6 U94/rep complements the replication of rep-deficient AAV-2 genome (Thomson et al., 1994). The U94/rep transcript is expressed at low levels during "in vitro" infection, suggesting that only small amounts of protein are required during productive viral replication or that it plays a primary role during latency (Rapp et al., 2000). Interestingly, U94/rep transcript is detected in "in vivo" latently infected peripheral blood mononuclear cells from healthy donors, in the absence of other viral mRNAs expressed during productive infection (Rotola et al., 1998; Yoshikawa et al., 2003). Furthermore, in vitro studies show that it might be involved in viral transcription and replication. U94/rep binds the human transcriptional factor TBP (Mori et al., 2000), binds ssDNA (unlike RCMV rep which binds also dsDNA; Dhepakson et al., 2002), inhibits transcription from HIV-1 LTR in T cell lines (Araujo et al., 1995) and suppresses transformation by H-*ras* (Araujo et al., 1995). In addition, T cell lines stably transformed with U94/rep are not permissive to HHV-6 lytic infection and inhibit HHV-6 origin of DNA replication in transient amplicon replication assays (Rotola et al., 1998; Turner et al., 2002). These results suggest that U94/rep might have a crucial role in the regulation of viral gene expression and replication and be important for the maintenance of viral latency.

However, the role of U94/REP in viral replication is not well documented due to the low yields of native protein from HHV-6-infected cells (Rapp et al., 2000) and to the difficulties in purifying a recombinant U94/REP protein. These problems have been circumvented by transfecting the U94/rep gene (Rapp et al., 2000; Rotola et al., 1998; Thomson et al., 1994; Turner et al., 2002) or by production of fusion proteins (Mori et al., 2000), but the availability of a purified U94/REP protein is a necessary requisite to characterize directly biological functions and to determine antigenic reactivity.

We therefore undertook the task of producing and purifying a recombinant U94/REP protein and to carry out functional studies. We succeeded in obtaining a >95% pure protein, which retained immunologic reactivity (Caselli et al., 2002). We show that the recombinant U94/REP protein inhibits the production of HHV-6 (both variant A and B) in infected T cells with a dose-dependent effect, confirming that it plays an important role in the replication cycle and could be a critical component in regulation of latency. Interestingly, U94/REP inhibits also other betaherpesviruses, HHV-7 replication in T cells and HCMV production in human fibroblasts, but has no effect on the alphaherpesvirus, herpes simplex virus (HSV) replication. Furthermore, we show that the mechanism of replication inhibition correlates with its ability to interact with single-stranded but not double-stranded DNA consistent with previous reports demonstrating U94/REP has a single-stranded DNA-binding protein (Dhepakson et al., 2002).

Results

Production and purification of the U94/REP protein

The full-length U94/rep gene (nucleotides 141,394 to 142,866) was excised by *Hin*dIII digestion from plasmid pSR2PH (Rotola et al., 1998) and cloned under the transcriptional control of the T5/lac promoter, obtaining the recombinant pQE-rep plasmid. The recombinant plasmid was transfected in M15 strain of *E. coli* host cells, and bacterial clones were selected after sequencing the insert to ensure that the correct coding sequence had been cloned in the plasmid vector. Following 1, 3 or 5 h of induction with 2 mM IPTG, samples were collected and analyzed by SDS-PAGE. The results showed that a protein with the predicted molecular weight of 56 kDa was expressed after 5 h of induction in pQE-rep transformed cells (Fig. 1A) and formed approximately 5% of the total bacterial proteins, as estimated by densitometric reading. The 56 kDa band was not present in the non-induced pQE-rep bacteria, as well as in the mock extract from bacteria containing only pQE vector sequences (not shown). The

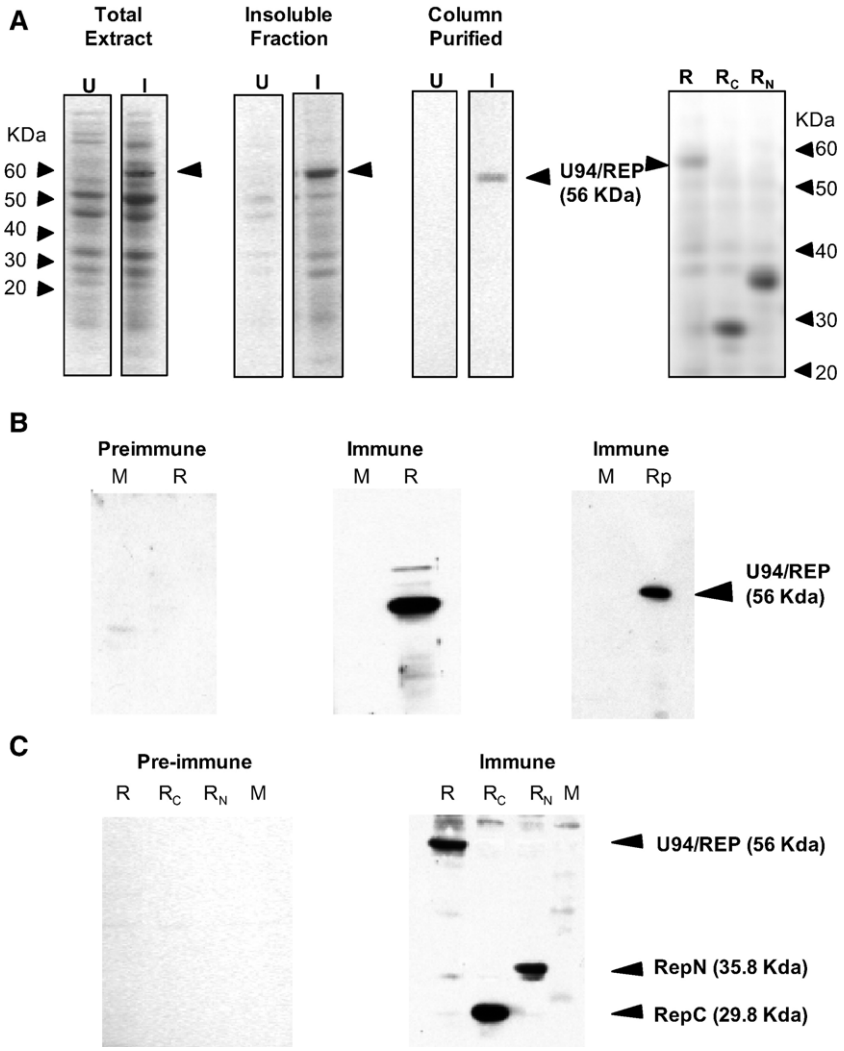

Fig. 1. Production and purification of U94/REP protein under denaturing conditions. (A) pQE-rep transformed M15 host cells were uninduced (U) or induced (I) with 2 mM IPTG for 5 h and analyzed by SDS-PAGE. The 56 kDa protein obtained in the insoluble fraction was then purified by ion exchange chromatography, as described in Materials and methods and subsequently analyzed by SDS-PAGE. Purification of the protein fragments of 29.8 and 35.8 kDa, corresponding to the carboxy-(R_C) and amino-terminal (R_N) regions of U94/REP is also shown. (B) Western blot assays were performed using a 1:200 dilution of pre-immune or immune rabbit serum, obtained by injection of pSR2pH plasmid as described in Materials and methods, and 5 μg/lane of crude recombinant U94/REP (R), purified U94/REP (Rp) or mock (M) as the antigens. (C) Western blot assays were performed as described for panel B, with 5 μg/lane of recombinant U94/REP (R), amino- or carboxy-terminal RepN or RepC subfragments (R_N, R_C) or mock (M) as the antigens.

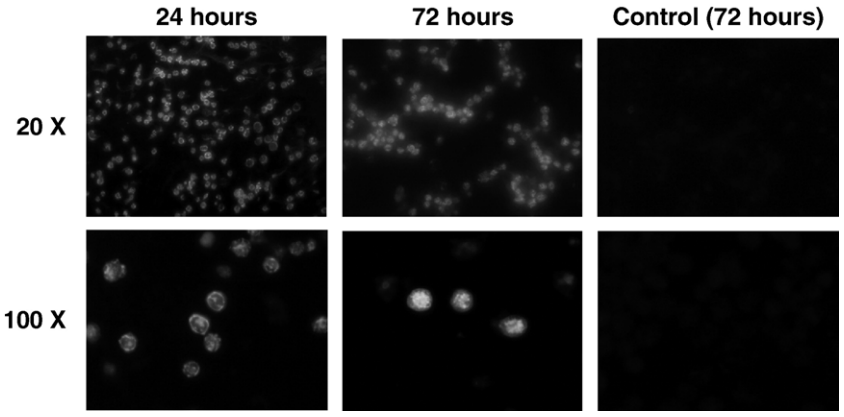

Fig. 2. Uptake and cellular localization of U94/REP protein. IFA analysis of treated J-Jhan cells was performed using mAb directed against the 6-His sequence located at the amino-terminus of the recombinant protein. Results refer to 24 and 72 h of incubation with 2 μg/ml of U94/REP protein.

protein was recovered mostly from the insoluble fraction, suggesting that it accumulated in bacterial inclusion bodies. To solubilize the crude U94/REP preparation, cell pellets were treated with lysis buffers containing increasing molar concentrations of urea. Complete solubilization of the crude U94/REP protein was achieved with 6 M urea. Therefore, purification of U94/REP was attempted by chromatography under denaturing conditions. The urea-solubilized protein was dialyzed to eliminate residues of Triton detergent then applied to a hydroxyapatite column and eluted with 500 mM phosphate buffer, obtaining a >95% pure protein (Fig. 1A). The yield of the purified protein was approximately 6 mg/l culture, as measured after refolding and aqueous solubilization achieved by serial dialysis in decreasing concentrations of urea. Also the amino (Aa 1–326) and carboxy (Aa 220–491) termini of U94/REP were cloned and produced using the same protocol (Fig. 1A).

Analysis of purified U94/REP proteins by Western blot assay and ELISA

The identity of the bacterial recombinant protein was confirmed by immunological assays. To this purpose, a polyclonal antiserum reacting specifically with the native HHV-6 U94/REP protein was obtained in rabbits by direct DNA immunization using plasmid pSR2PH as the immunogen.

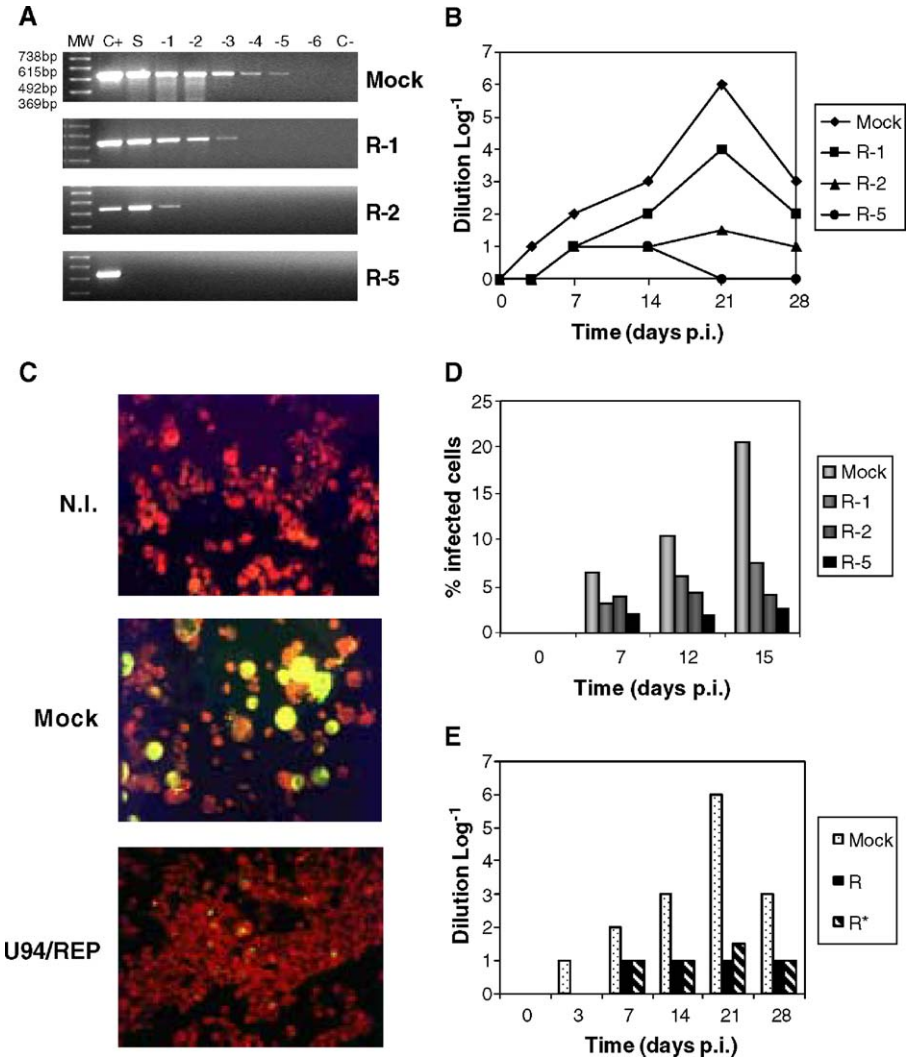

Fig. 3. Biological activity of recombinant U94/REP protein on HHV-6 variant A infection. Susceptible J-Jhan T cells were cultured and infected as described in Materials and methods with HHV-6 variant A (strain U1102). Cell samples were collected at 1-week intervals and analyzed by semiquantitative PCR. (A) Semiquantitative PCR amplification of HHV-6 DNA, from samples collected 21 days post-infection. Serial ten-fold dilutions (−1 to −6 lanes) of template DNA extracted from cell samples (S) were amplified in the genomic region of HHV-6 U42. DNA from HHV-6A infected J-Jhan cells at full cytopathic effect was used as a positive control (C+). The DNA extracted from uninfected cells was also used as the negative control (C−). Mock shows HHV-6-infected cells in the presence of 5 μg/ml of mock bacterial lysate, R1–R5 are HHV-6-infected cells in the presence of 1, 2 or 5 μg/ml of U94/REP protein. (B) The plot shows the time course of HHV-6 replication, expressed as the maximum dilution which resulted positive for the presence of HHV-6A DNA. (C) Immunofluorescence with monoclonal antibody to HHV-6 gp116 of uninfected (N.I.) and HHV-6-infected J-Jhan cells in the presence of mock lysate (Mock) or of 2 μg/ml of U94/REP (U94/REP). The results shown correspond to cell samples collected 21 days p.i. (D) Time course of the results of IFA assay. Results are expressed as positive cells/total cells (%), and each value represents the mean of three different samples. (E) Time course of HHV-6 infection of J-Jhan cells in the presence of 2 μg/ml of U94/REP, added before (R) or after (R*) infection.

Animals were injected by intramuscular route and subsequently boosted subcutaneously and intradermally, obtaining specific anti-U94/REP antisera.

The immunological reactivity of the recombinant proteins was analyzed by Western blot (Fig. 1B). Proteins from induced bacteria transformed either with pQE-rep or control pQE30 were separated by electrophoresis and transferred onto nitrocellulose filters. Blots were incubated with the polyclonal rabbit antiserum raised against U94/rep DNA diluted 1:200 and developed with the addition of chemiluminescent substrate. The results show that the U94/REP fusion protein is specifically recognized by the rabbit antiserum obtained by DNA immunization, resulting in a specific reactive band absent in the mock bacterial lysate and not reacting with pre-immune rabbit serum (Fig. 1B). The purification procedure did not modify the immuno-reactivity of the purified protein, as shown by the presence of a single reactive band with the expected molecular weight. The rabbit anti-U94/REP polyclonal antiserum specifically recognized also protein subfragments corresponding to the amino- and carboxy-terminal regions of U94/REP (RepN and RepC), resulting in reactive bands of 35.8 and 29.8 kDa respectively (Fig. 1C).

The U94/rep gene product obtained in bacteria reacted also in ELISA, as shown by the reactivity observed with the immune rabbit antiserum (OD_{492nm} = 0.382) but not with the pre-immune serum (OD_{492nm} = 0.061). The specificity of binding was further confirmed by absence of reactivity of immune serum against mock coated plates (OD_{492nm} = 0.048).

Analysis of U94/REP uptake and cellular localization

The ability of the recombinant protein to be internalized by human cells was analyzed by IFA, using a mAb specifically recognizing the stretch of histidines located at the amino-terminus of the fusion protein. J-Jhan cells were cultured with 1, 2 or 5 μg/ml of U94/REP and analyzed 24 and 72 h later. The results show that U94/REP was internalized by cells, as indicated by a bright generalized cell staining after 24 h, and accumulated in the cell nucleus at 72 h, indicating a specific nuclear targeting of the protein (Fig. 2).

Analysis of U94/REP biological activity

To ascertain whether the recombinant protein produced in *E. coli*, purified under denaturing conditions and refolded, maintained biological activity, we analyzed its effect on HHV-6A infection in T cell lines, based on the previous observation that T cell clones transformed with U94/rep gene are resistant to HHV-6 lytic infection (Rotola et al., 1998). Aliquots of permissive J-Jhan cells were cultured in the presence of different concentrations of refolded purified U94/REP or mock lysate (control) and subsequently infected with a standardized inoculum of HHV-6 A (strain U1102). The titer of the purified cell-free viral stock was 10^3 $TCID_{50}$/ml. Cells infected with HHV-6 in the absence of U94/REP developed cytopathic effect between day 3 and 7 and were completely lysed between 14 and 21 days after infection. The relatively long time to develop complete cell lysis is to be ascribed to the low MOI, due to the low infectious titer of purified viral stock. Cells infected in the presence of U94/REP developed little or no cytopathic effect, and 4 weeks after infection, no cell lysis was observed, with all concentrations of U94/REP. Samples were collected at 0, 3, 7, 14, 21 and 28 days p.i. and

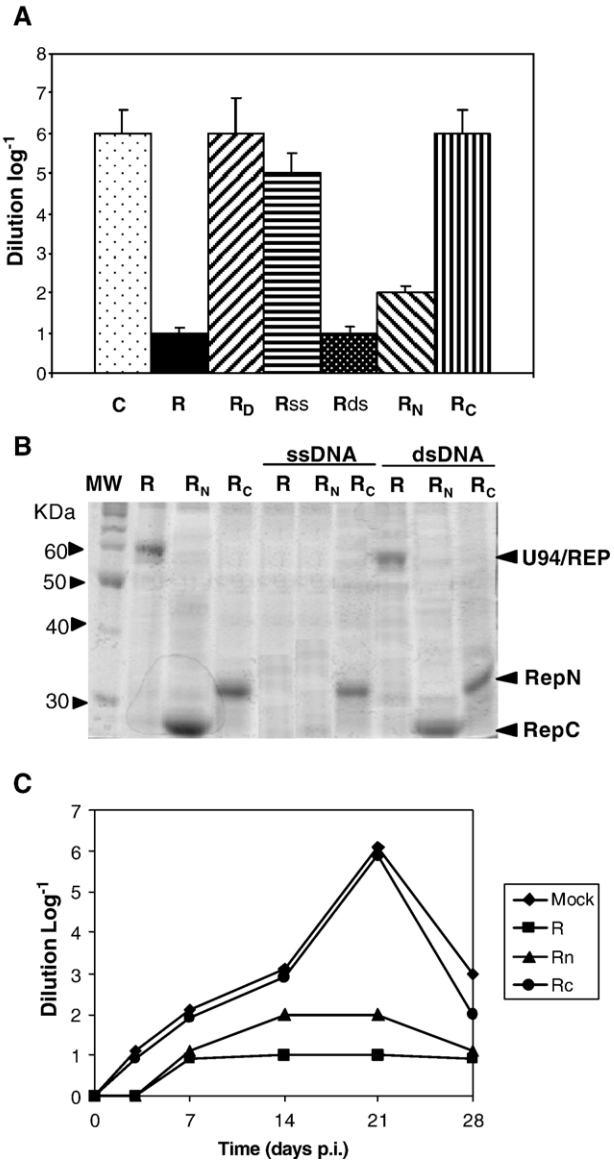

Fig. 4. Biological activity of U94/REP protein on HHV-6 variant A infection. (A) J-Jhan cells were cultured and infected as described. Cell samples were collected at 1-week intervals and analyzed by semiquantitative PCR of HHV-6 DNA, performing serial ten-fold dilutions (−1 to −6) of template DNA. The results show U42 amplification performed on the sample of day 21, corresponding to the peak of virus production, treated with 2 μg/ml protein. Results are expressed as the maximum dilution which resulted positive for the presence of HHV-6A DNA; control (C), U94/REP (R), heat denatured U94/REP (R_D), U94/REP plus ssDNA (Rss), U94/REP plus dsDNA (Rds), RepN amino-terminal region (R_N), RepC carboxy-terminal region (R_C). (B) Results of SDS-PAGE analysis of eluate fractions after ssDNA or dsDNA-affinity column chromatography performed as described in Materials and methods. (C) Time course of HHV-6 replication in the presence of U94/REP (R), N-terminal domain (R_N) and C-terminal domain (R_C), expressed as the maximum dilution which resulted positive for the presence of HHV-6A DNA.

evaluated for the presence and expression of HHV-6 by PCR and rtPCR. The recombinant protein produced in bacteria inhibited HHV-6 replication in J-Jhan-infected cells in a dose-dependent manner, and both viral DNA and rates of expression of viral functions were significantly decreased (Figs. 3A–B). U94/REP-treated cells had viral DNA loads up to 6 logs lower than control cells infected with HHV-6 in the presence of mock bacterial lysate, as shown by semiquantitative PCR analyses performed on U42 gene. Extinction of the signal after amplification of β-actin occurred at the same dilution for all samples (not shown), showing that the same amount of material has been analyzed for all samples. Upon removal of U94/REP from culture medium, cells returned fully susceptible to HHV-6 infection within 48 h (not shown), confirming that resistance to virus replication is associated to the presence of U94/REP. Similar results were obtained performing PCR analysis on different genomic regions (U31 and U94—data not shown) and by rtPCR, analyzing transcripts from IE and E genes, respectively, U42 and U31 (not shown). No difference in the cellular growth rate and viability was observed between control cells and cells treated with U94/REP, as shown by Trypan Blue cell counting at different times p.i. and by analysis of cytotoxicity by MTT assay (data not shown). The results were confirmed by IFA, using a commercial mAb against gp116 (late antigen). As shown in Figs. 3C–D, expression of virus antigens and cytopathic effect was significantly inhibited in cells treated with U94/REP compared to control mock-treated cells infected with the same amount of virus, confirming that U94/REP inhibits HHV-6 replication.

To determine whether U94/REP interferes with virus entry, resulting in inefficient infection, or acts after adsorption, inhibiting viral replication, U94/REP was added to HHV-6-infected cells only after removal of the virus inoculum. Also in this instance HHV-6 replication and yield were significantly reduced (Fig. 3E). The degree of inhibition was similar to that observed when infection takes place in the presence of U94/REP, suggesting that the viral protein acts after virus entry.

To confirm specificity and investigate mode of action, HHV-6 infection experiments were repeated using equal amounts of

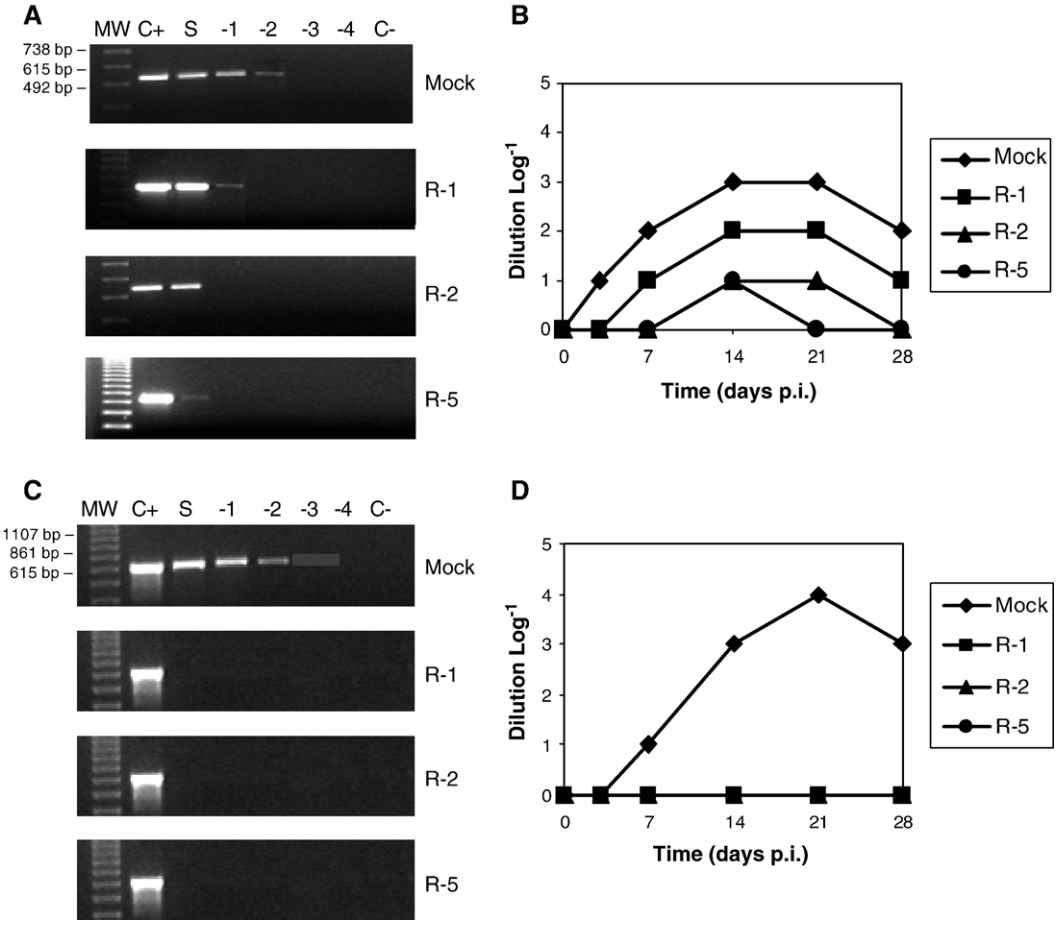

Fig. 5. Biological activity of recombinant U94/REP protein upon HHV-6 variant B (A, B) and HHV-7 (C, D) infection of susceptible Sup T1 cells. Cells were infected as described in Materials and methods, respectively, with HHV-6 variant B (strain CV) or HHV-7 (strain CZ). Cell samples were collected at 1-week intervals and analyzed by semiquantitative PCR and rtPCR. Mock results refer to 5 μg/ml of mock bacterial lysate; identical results were obtained with the other amounts used. (A and C) Semiquantitative PCR amplification of gene U42 for HHV-6B and HHV-7, respectively. Positive controls (C+) were represented by cDNA from HHV-6B- or HHV-7-infected Sup T1 cells collected at complete cytopathic effect. Amplification reactions were performed by using 1:10 serial dilutions (−1 to −6 lanes) of the DNA template extracted from sample cells (S). (B and D) The diagrams summarize the results, expressed as the maximum dilution which resulted positive for the presence and transcription respectively of HHV-6B and HHV-7.

U94/REP either heat denatured or preincubated with ssDNA or dsDNA or with the two amino- and carboxy-terminal RepN and RepC protein subfragments. The results, summarized in Fig. 4A, show that heat denaturation abolished U94/REP activity upon HHV-6 replication, suggesting that the folding of the protein is important for its action. One important molecular characteristic of U94/REP is the ability to bind ssDNA and not dsDNA, as originally described by Dhepakson et al. (2002). This is distinct from rCMV rep which binds both ds and ssDNA (van Cleef et al., 2004). Our preparation of U94/REP retains this characteristic, binding ssDNA but not dsDNA (Fig. 4B). This DNA-binding activity is required for the U94/Rep inhibition of replication since preincubation of the protein with ssDNA, but not with dsDNA, neutralized the inhibitory activity of U94/REP (Fig. 4A). The C-terminal domain showed no inhibition of replication, further confirming specificity, whereas the N-terminal domain (RepN) retained inhibitory activity (Fig. 4A), suggesting that the active domain is encoded by the 5′ end of the gene. The activity of N-terminal domain, but not of C-terminal, was shown also by time course experiments (Fig. 4C). Interestingly, only the N-terminal domain retained the ability to bind ssDNA (Fig. 4B).

The activity of U94/REP was tested also on the distantly related alphaherpesvirus, HSV, as well as the other human betaherpesviruses: HHV-6 variant B (strain CV), HHV-7 (strain CZ) and HCMV (Towne strain). Samples were collected and analyzed as described in Materials and methods with MTT assays performed on the cells treated with U94/REP to confirm the absence of non-specific cytotoxic effects. While no differences in virus production were shown in the presence or absence of U94/REP during HSV-1 (strain F) infections of permissive Vero cells (not shown), there were marked effects on the betaherpesviruses. Results of HHV-6B and HHV-7 infection analyses are summarized in Fig. 5 and show that U94/REP inhibits with a dose-dependent effect on both HHV-6B and HHV-7 replication. Similarly, IFA analysis of HCMV infection (Fig. 6) shows that U94/REP treatment severely impairs virus replication, inhibiting expression of IE antigens (IE-1 and 2), early (ppUL44, not shown) and late proteins (pp65 and gB).

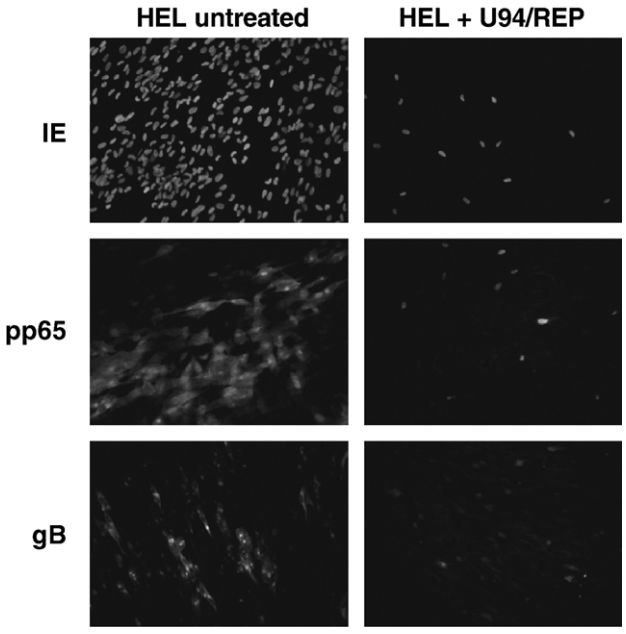

Fig. 6. Biological activity of U94/REP protein upon HCMV infection on permissive HEL cells. Cells were infected as described in Materials and methods and collected at 5 days p.i. for IFA analysis, using anti-IE-1, anti-pp65 and anti-gB antibodies. Right column: untreated HEL cells infected with HCMV (m.o.i. 0.05 PFU/cell). Left column: cell infected in the presence of U94/REP (3 μg/ml).

Discussion

The genome of HHV-6 encodes U94/rep, a gene absent in all other members of the human herpesvirus family, with a striking homology to the AAV-2 rep 68/78 gene. rep68/78 plays a pivotal role in AAV-2 replication (Berns and Giraud, 1996) as it is involved in AAV-2 DNA integration in the infected cell genome, it is required for viral DNA replication, and it modulates gene expression. Furthermore, it prevents oncogenic transformation. HHV-6 U94/rep is more closely related to AAV-2 rep than are the homologous proteins encoded by other members of the parvovirus family (Thomson et al., 1991) and possesses at least some of the functional properties of the AAV-2 rep. In fact, HHV-6 U94/rep complements replication of rep-deficient AAV-2 genomes (Thomson et al., 1994), regulates gene expression (Araujo et al., 1995) and can prevent oncogenic transformation (Araujo et al., 1995). However, the precise function of U94/rep in HHV-6 biology and replication is still largely undetermined. U94/REP can regulate gene expression, as suggested by the observations that it binds to single-stranded DNA (Dhepakson et al., 2002) and to human transcription factors (Mori et al., 2000). It has been suggested that U94/REP might be involved in viral latency. In fact, U94/rep mRNA is detected in PBMCs of latently infected normal donors (Rotola et al., 1998; Yoshikawa et al., 2003). The potential involvement in viral latency is suggested also by the fact that U94/rep expression in stably transformed cell lines reduces HHV-6 DNA and RNA production (Rotola et al., 1998) and that it inhibits replication of HHV-6 “amplicons” containing the HHV-6 origin of replication (Turner et al., 2002).

Studies on the function of this unique gene have been hindered by the lack of a convenient source of U94/REP. U94/REP is expressed in HHV-6-infected cells only at low levels (Rapp et al., 2000), and although high expression can be obtained by in vitro transcription–translation, a range of truncated forms are produced (Gompels, unpublished results); furthermore, transformed eukaryotic cell lines transcribing the U94/rep gene were easily obtained, but it has not been possible to produce and purify the protein, due to the low levels of expression (Rotola et al., 1998; Caselli and Di Luca, unpublished). Previous reports were obtained transfecting the U94/rep gene or producing a fusion protein (Mori et al., 2000; Rapp et al., 2000; Rotola et al., 1998; Thomson et al., 1994; Turner et al., 2002). Instead, we have produced in *E. coli* and purified the full-length U94/REP protein. The purified protein

reacts specifically in ELISA and immunoblotting with a polyclonal anti-U94/REP rabbit serum obtained by DNA immunization. The short poly-(His) sequence present in the fusion protein does not affect serological detection; therefore, it was not necessary to remove this tail after purification. The specific reactivity observed in Western blots was confirmed using human sera from HHV-6-positive individuals (Caselli et al., 2002), suggesting that the protein is immunogenic and is expressed during in vivo infection.

Here, the biological effect of U94/REP was studied by incubating infected cells in medium containing the recombinant protein. Exogenous U94/REP is internalized by human cells and targets specifically to the nucleus (Fig. 2), in accordance with its possible role in DNA replication and expression. U94/REP has no effect on viral entry but inhibits significantly HHV-6 production (Fig. 3), and the block takes place before DNA replication, as shown by semiquantitation of viral DNA and IE/E mRNAs. The function of U94/REP is dependent on the protein conformation, being abolished by heat denaturation, and is mediated by single-strand DNA-binding activity (Figs. 4A, B). Most likely, the active domain is contained in the N-terminal fragment, as shown by the lack of activity of the C-terminus.

Interestingly, the inhibitory effect of U94/REP is not limited to HHV-6 but extends to the other human betaherpesviruses HCMV and HHV-7. Also in the case of HCMV, the inhibition takes place at an early stage of replication. Furthermore, during the immediate-early phases of infection, the matrix protein encoded by UL83 (pp65) localizes in the nucleus as a component of viral inoculum, and, after DNA replication, it is present mostly in the cytoplasm. In the presence of U94/REP, pp65 was exclusively present in the nuclei of HCMV-infected cells, suggesting that DNA replication was blocked (Fig. 6). The activity of U94/REP is specific for betaherpesviruses, and the alphaherpesvirus, HSV-1, replication is not affected. This is also consistent with the observation that HHV-6 cannot recognize the origin binding protein (OBP) sites required for HSV replication, although it shares a similar strategy in encoding an OBP (Inoue and Pellett, 1995). It is possible that U94/REP has several targets. It inhibits the viral origin of DNA replication, as shown by transient in vitro replication assays (Turner et al., 2002), and this is further shown here with the direct effect of U94/REP protein on virus replication which correlates with its ssDNA-binding activity. It also represses viral transcription, as shown by a significant reduction of viral IE/E mRNAs. This could be mediated by interaction with cellular factors, as in the case of AAV-2 rep68/78, that downregulates AP-1-dependent transcription (Prasad et al., 2003).

The inhibitory effect of U94/REP on viral replication strengthens the notion that it might have a role in the establishment and maintenance of viral latency, as originally suggested by the detection of U94/rep transcripts in PBMCs of latently infected healthy adults (Rotola et al., 1998). Furthermore, high levels of antibody to U94/REP have been detected in some multiple sclerosis patients, suggesting differences in latency regulation, though this could be contributed by underlying immune defects in these patients (Caselli et al., 2002). As a general consideration, the latency of herpesviruses does not depend on the presence of rep homologues, and, among the many herpesviruses so far characterized, only 2 betaherpesviruses, HHV-6 and the rat cytomegalovirus (RCMV), encode a rep homologue, with similar genomic position and orientation (Thomson et al., 1994; van Cleef et al., 2004). However, the RCMV rep (r127) is dispensable for virus replication and has no significant effect on virus production, although effects on latency were not examined (van Cleef et al., 2004). Interestingly, RCMV r127 is truncated at the N-terminus, and the inhibitory activity of HHV-6 U94/REP resides in the N-terminus. Furthermore, RCMV rep r127 differs from HHV-6 U94/REP in that it has broad DNA-binding activity to both ss and dsDNA binding, whereas U94/REP is specifically an ssDNA-binding protein (Dhepakson et al., 2002). Of note, AAV rep which shares sequence similarity with HHV-6 rep can be complemented by HHV-6 U94/rep and also can inhibit herpesvirus DNA replication using a domain which maps to the N-terminus (Kleinschmidt et al., 1995; Thomson et al., 1994). It has also been shown that HHV-6 latency is characterized by a transcription pattern similar to that of HCMV, with transcripts from the E1/E2 regions (Kondo et al., 2002). Therefore, it is possible that HHV-6 latency does not depend exclusively on U94/rep. Nevertheless, U94/rep is highly conserved in HHV-6 variants, is transcribed in latently infected PBMCs and inhibits betaherpesviruses replication. These observations point to a specific role for U94/REP in the regulation of virus production and, combined with the observation that co-reactivations of betaherpesviruses are frequent in immunosuppressed patients, suggest that it might be an interesting candidate for the development of new antiviral agents specific for betaherpesviruses.

The origin of rep genes in HHV-6 and RCMV is completely unknown. HHV-6 U94/rep appears to have a different evolutionary conserved role from RCMV rep as in HHV-6 strains U94/rep genes are highly conserved whereas RCMV rep is absent in some RCMV strains (Voigt et al., 2005). However, the rep genes in HHV-6 and RCMV have similar genomic locations and orientations, suggesting that they might have diverged from a common origin. This suggests that an ancestral parvovirus may have integrated in the genome of a betaherpesvirus progenitor during coinfection and rep sequences were lost after phylogenetic divergence, except when they developed a novel function in the herpesviral host (van Cleef et al., 2004). Moreover, in the case of the closely related HHV-6 and HHV-7, the fact that HHV-7 can replicate the HHV-6 ori in trans replication assays but HHV-6 cannot replicate the HHV-7 ori while HHV-7 which lacks U94/rep can reactivate HHV-6 (Katsafanas et al., 1996) may also point to a role here of the HHV-6 replication inhibitor U94/REP in the regulation of replication and latency (Steeper et al., 1990; van Loon et al., 1997). Thus, in the case of HHV-6, the novel functions gained might be represented by a tight control of latency and a reduction of viral replication, possibly leading to the natural selection of less virulent strains to favor long-term survival in the host.

Materials and methods

Viruses and cells

Cell-free virus inocula were obtained as previously described: HHV-6 variant A (strain U1102) was grown and analyzed in the J-Jhan cell line (Rotola et al., 1998); HHV-6 variant B (strain Z29) and HHV-7 (strain CZ; Portolani et al., 1995) were grown and analyzed in the Sup T1 cell line (Menegazzi et al., 1999); HSV-1 (strain F) was grown and analyzed in the epithelial Vero and in the T lymphoid J-Jhan cell lines (Caselli et al., 2000). HCMV (Towne strain) was grown on human embryonic lung fibroblasts (HEL; Nakamura et al., 1988).

Both T lymphoid J-Jhan and Sup T1 cell lines were grown in suspension in RPMI 1640 supplemented with 10% fetal calf serum (FCS). Adherent Vero and HEL cells were respectively cultured in DMEM and MEM supplemented with 10% FCS.

Plasmids construction

The full-length U94/rep gene was obtained by PCR amplification of ORF U94 of HHV-6 variant B, as previously described (Rotola et al., 1998). The U94/rep gene from HHV-6B R1 strain (Dewhurst et al., 1992), cloned in the pSR2PH vector, was excised by *Hin*dIII digestion and subcloned into bacterial pQE30 vector (Qiagen), obtaining the recombinant plasmid pQE-rep. The gene was cloned in frame with a stretch of six histidine residues $(His)_6$ at the amino-terminus, under the control of a T5 promoter/lac operator. The recombinant plasmid was used to transform the strain M15 of *E. coli* host cells, harboring the pREP4 plasmid, which contains the lacI gene coding for lac repressor. Two other plasmids were constructed, containing the amino-terminal (RepN) and the carboxy-terminal (RepC) regions of U94/rep gene. These regions were obtained by *Pst*I and *Eco*RV–*Hin*dIII digestion of pSR2PH vector, resulting in two fragments of 978 bp and 813 bp which were subcloned in pQE30 vector.

Upon addition of isopropylthiogalactoside (IPTG), the T5/lac promoter is activated, resulting in high yield production of the fusion proteins cloned in pQE30.

Production and purification of U94/REP protein

Bacterial M15 host cells transformed with plasmid pQE-rep were grown at 37 °C in LB medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin up to OD_{660nm} = 0.4–0.6 and subsequently induced with 2 mM IPTG. After 1, 3 and 5 h of induction at 37 °C, samples were collected by centrifugation and analyzed for the presence of the recombinant proteins. Cell pellets from 500 ml culture were suspended in 50 ml lysis buffer (50 mM Tris–HCl pH 8, 2 mM EDTA, 1 mM DTT, 1 μg/ml aprotinin, 1 mM PMSF, 100 μg/ml lysozyme and 1% Triton X-100). After incubation for 30 min at room temperature, the suspension was cleared by sonication (three cycles of 15 s with a 15 s interval) and centrifuged in a microfuge for 15 min at 13,000 rpm at 4 °C. Samples from supernatant (soluble fraction), pellet (insoluble fraction) and total lysate were analyzed by SDS-PAGE. U94/REP protein was recovered mostly from the insoluble fraction, thus samples were solubilized in lysis buffer containing increasing molar concentration of urea (2, 4, 6, 8 M). Each fraction was analyzed in SDS-PAGE and Western blot. Complete solubilization of the protein was achieved with lysis buffer supplemented with 6 M urea. The fraction containing the recombinant protein was dialyzed to eliminate residues of Triton X-100 and purified under denaturing conditions by hydroxyapatite chromatography, using lysis buffer in a phosphate gradient. The sample was applied to the column after equilibration with lysis buffer without Triton, pH 6.8. The resin was washed with a 10–200 mM sodium phosphate buffer, and the protein was eluted with 500 mM phosphate buffer. Each fraction was examined by spectroscopy at 280 nm and subsequently analyzed by SDS-PAGE and Western blot. Endotoxin levels were tested and were below 0.5 EU/μg protein in all assays performed. The same procedure was applied to obtain RepN and RepC protein fragments, resulting in two peptides of 35.8 and 29.8 kDa respectively. Mock preparations were obtained from M15 *E. coli* cells transformed with the pQE30 vector alone, extracted as described for pQE-rep transformed cells. Briefly, cells were cultured for 5 h in the presence of 2 mM IPTG, then extracted and solubilized with lysis buffer containing 6 M urea, and finally refolded in aqueous buffer. Mock lysate was used at the same protein concentration as purified U94/REP protein.

Production of polyclonal anti-U94/REP serum

To obtain a specific anti-U94/REP serum, rabbits were immunized by DNA vaccination, using as immunogen the plasmid pSR2PH, containing HHV-6 U94/rep. Plasmid DNA was prepared as previously described (Caselli et al., 1999, 2000) and suspended 1 mg/ml in sterile phosphate buffer (PBS; 137 mM NaCl, 3 mM KCl, 80 mM Na_2HPO_4, 1 mM NaH_2PO_4, pH 7.4). Animals were injected with 300 μg of pSR2PH DNA by intramuscular route and subsequently boosted (with 2-week intervals) with the same dose of DNA, once subcutaneously and twice intradermally in the ear pinna. One week before intramuscular immunization, animals were injected with 100 μl of 0.5% bupivacaine solution, with the aim to increase antigen capture and expression at the site of DNA injection (Caselli et al., 1999, 2000). Blood was collected from the ear vein before (pre-immune control serum) and after (immune serum) immunizations. Serum was obtained by centrifugation of coagulated blood and used in Western blot assay and ELISA to determine the presence and identity of U94/REP protein.

Western blot assay and ELISA

To assess the identity of the recombinant U94/REP protein, specific Western blot and ELISA were developed. The rabbit antiserum obtained by DNA immunization was utilized as the primary antibody. Briefly, for Western blot, the same amount of U94/REP protein, RepN and RepC protein subfragments or

mock lysate (used as the control antigen) was separated by SDS-PAGE and electrically transferred onto nitrocellulose paper with transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). Blots were incubated 1.5 h in a saturation buffer consisting of 5% dehydrated non-fat milk in TBS (10 mM Tris–HCl, pH 7.4, 150 mM NaCl). After three washings of 10 min each with TBS containing 0.5% Tween 20 (TBS-T), nitrocellulose filters were incubated for 1 h in fresh TBS-T containing 5% dehydrated milk and the rabbit polyclonal antiserum diluted 1:200. Following three additional washings in TBS-T, blots were incubated for 2 h with the appropriate dilution of a horseradish-peroxidase (HRP)-labeled goat anti-rabbit IgG (Roche Molecular Biochemicals) in TBS-T plus 5% dehydrated milk. Blots were further washed three times with TBS-T and then developed with the addition of a chemiluminescent HRP substrate (SuperSignal West Pico Chemiluminescent Substrate, Pierce), according to the manufacturer's protocol.

For ELISA, immunoplates (Nunc) were coated overnight at 4 °C with 5 μg/ml of purified recombinant U94/REP, or mock lysate, suspended in 0.05 M sodium-bicarbonate buffer (pH 9.6). Excess of antigen was eliminated by three washings with PBS containing 0.05% Tween 20 (PBS-T). A saturation step was performed by incubating plates for 90 min at 37 °C with 200 μl/well of a PBS solution containing 10 mM $CaCl_2$ and 5 mM $MgCl_2$ (PBS-C) and 3% of bovine serum albumin (BSA; Sigma). After three washings with PBS-T, 100 μl of the rabbit antiserum obtained by DNA immunization, serially diluted in saturation buffer (from 1:25 to 1:200), was added and tested in duplicate. Pre-immune rabbit serum was used as negative control. Incubation was performed for 90 min at 37 °C. Plates were washed three times with PBS-T then 100 μl of horseradish-peroxidase-labeled goat anti-rabbit IgG (Roche Molecular Biochemicals), diluted 1:3000 in PBS-T plus 1% BSA, was added to each well. Incubation was performed for 90 min at room temperature. Following three additional washings, 100 μl/well of ABTS substrate (Roche Molecular Biochemicals) was added for 45 min at room temperature. The absorbance was measured at 405 nm. Absorbance values higher than the mean control plus three standard deviations (SD) were considered positive.

Analysis of U94/REP biological activity

The biological activity of recombinant U94/REP protein was assayed by testing its effect upon the replication of different herpesviruses in experiments of in vitro infection. For HHV-6A, 10^7 J-Jhan cells were cultured for 24 h in complete medium supplemented with 0, 1, 2 or 5 μg/ml of recombinant U94/REP or mock lysate (control) then centrifuged and infected with a standardized amount of HHV-6A (strain U1102) from a stock of 10^3 $TCID_{50}$/ml, as previously described (Rotola et al., 1998). Following 1 h of infection, virus inoculum was removed, and cells were suspended in fresh medium containing 0, 1, 2 or 5 μg/ml of U94/REP or mock lysate. Alternatively, the recombinant protein was added only after the removal of virus inoculum. Cell samples were collected at 0, 3, 7, 14, 21 and 28 days post-infection (p.i.) and extracted as previously described for PCR and rtPCR analyses (Menegazzi et al., 1999; Rotola et al., 1998). The same experiments were also performed with equal amounts of the RepN and RepC protein subfragments or with U94/REP protein denatured by boiling at 100 °C for 10 min or U94/REP preincubated for 30 min at 37 °C with 20 μg/ml of calf thymus single-stranded or double-stranded DNA (ssDNA or dsDNA; Sigma).

HHV-6B and HHV-7 infections were performed on Sup T1 cells following the same procedure. For HCMV infection, viral absorption was performed on 70–80% confluent monolayers of HEL cells at a m.o.i. of 0.05 PFU/cell for 1 h, then virus inoculum was removed and fresh medium containing 0, 1, 2 or 5 μg/ml of U94/REP or mock lysate was added. Virus production was evaluated 5 days after infection by immunofluorescence assay.

HSV-1 infection was performed both in J-Jhan and in Vero cells, using an m.o.i. of 0.01 and 0.001 PFU/cell. Briefly, 10^6 J-Jhan cells or 5×10^5 Vero cells (seeded in 6-well plates) were pre-treated for 24 h with U94/REP as described for HHV-6 and infected with 0.01 or 0.001 PFU/cell of HSV-1 (strain F) for 1 h in PBS with 1% FCS. Virus inoculum was removed, and fresh complete medium with 0, 1, 2 or 5 μg/ml of U94/REP or mock lysate was added. After 48 h of incubation, virus production was evaluated by lysing cells and culture supernatant as described (Rotola et al., 1998) and titrating lysates on Vero cells in the presence of 0.2% of gamma globulins.

U94/REP DNA-binding assay

The DNA-binding ability of U94/REP was evaluated by DNA-affinity column chromatography. Briefly, 50 μg of recombinant protein was applied to a 0.5 ml column of rehydrated ssDNA or dsDNA-cellulose (Sigma). After 1 h of incubation at 4 °C, the resin was washed with 1 ml of buffer. Aliquots of the input, flowthrough and wash fractions were then analyzed by SDS-PAGE and Western blot.

PCR analyses

The presence and transcription of HHV-6A, HHV-6B and HHV-7 in infected cells were analyzed by PCR and rtPCR amplification of the following genes: U94, U42 and U31 for HHV-6 A and B (Rotola et al., 1998) and U89/90, U42 and U31 for HHV-7. PCR amplification was performed using respectively 100 ng of total DNA or 200 ng total RNA extracted from infected or uninfected cells. Amplification of the house-keeping β-actin gene was used as a control.

Immunofluorescence assays

HHV-6 and HCMV infections were also analyzed by specific immunofluorescence assays (IFA). For HHV-6, 10^5 cells were spotted onto glass slides and fixed by cold acetone for 30 min at −20 °C. Slides were air-dried and kept at −20 °C until use. For assay, slides were rehydrated by washings in

PBS, incubated with mouse monoclonal antibodies (mAb) directed against glycoprotein gp116 (late antigen) of HHV-6 A and B (ABI, Columbia, MD, USA) diluted 1:100 in PBS for 35 min at 37 °C in a humidified chamber. Slides were washed twice with PBS for 10 min, once for 1 min with tap water and once for 1 min with distilled water, and incubated with secondary antibody, FITC-conjugated goat-anti-mouse IgG (Sigma, St. Louis, Missouri, USA) diluted 1:50 in PBS for 35 min at 37 °C. After washings as described, slides were stained with 0.01% Evan's Blue for 2 min, washed in distilled water and finally mounted with 50% glycerol in PBS for fluorescence microscope observation. For HCMV, infected HEL cells were fixed 5 days after infection with cold methanol–acetone (3:1) for 30 min at −20 °C, air-dried then incubated for 1 h at 37 °C in a humid chamber with the following primary mAbs diluted in PBS: anti-IE-A (clone E13) and anti-pp65 (Clones 1C3+AYM-1; Argene BIOSOFT, France), diluted 1:20; anti-ppUL44 (Clone 1202, Goodwin Institute for Cancer Research, Plantation, Florida) diluted 1:200; anti-gB, kindly obtained from M. Mach (Erlangen, Germany), undiluted. A secondary FITC-conjugated Goat anti-mouse antibody (Cappel, Organon Teknika Corp., Chester, PA, USA) was used diluted 1:50.

Uptake and cellular localization of U94/REP protein was analyzed by a specific IFA using an FITC conjugate mAb directed against the stretch of six histidine residues $(His)_6$ located at the amino-terminus of the recombinant protein (Penta-His Alexa Fluor, FITC conjugate, Qiagen). J-Jhan cells were cultured in complete RPMI medium containing 0, 1, 2 or 5 μg/ml of U94/REP or mock lysate for 24 and 72 h. Cell samples were washed twice in PBS, spotted onto glass slides, fixed by 4% paraformaldehyde for 10 min and permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature. U94/REP was revealed by incubation with the anti-His mAb diluted 1:2000 in PBS plus 1% BSA at 4 °C overnight. Slides were then washed four times in PBS and mounted with 50% glycerol in PBS for microscope observation. All samples were observed under a UV light microscope (Nikon Eclipse E600) equipped with a digital camera (DMX 1200).

Cytotoxicity assay

The toxicity of U94/REP recombinant protein on treated cells was measured by the MTT assay. For suspension cultures, cells were cultivated for 0, 3, 7, 14, 21 and 28 days in complete medium containing 0, 1, 2 or 5 μg/ml of U94/REP or mock lysate. At the indicated time points, 10^5 cells per well were seeded in a 96-well plate in 200 μl medium, incubated for 24 h and then supplemented with 25 μl/well of stock MTT solution (5 mg/ml; Sigma). Cells were further incubated at 37 °C for 4 h, then the culture medium was carefully removed by plate centrifugation, and 100 μl of DMSO was added to each well for 1 h at 37 °C. The absorbance of samples was measured at a wavelength of 570 nm with a microtiter plate reader. Each assay was performed in triplicate. Adherent cells were directly seeded in 96-well plates (10^4 cells per well in 200 μl volume) and incubated for 48 h with 0, 1, 2 or 5 μg/ml of U94/REP or mock lysate. After 48 h, cell samples were processed and assayed as described for suspension cells.

Acknowledgments

This work was supported by the Italian Ministry of Health (Istituto Superiore di Sanità, AIDS project), and MIUR. U.A. Gompels thanks the Royal Society for support. We thank Linda M. Sartor for English revision of the manuscript.

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