The U28 ORF of human herpesvirus-7 does not encode a functional ribonucleotide reductase R1 subunit

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Herpesvirus ribonucleotide reductases, essential for the de novo synthesis of viral DNA, are composed of two non-identical subunits, termed R1 and R2. The U28 ORF from human herpesvirus-7 has been classified, by sequence comparisons, as a homologue of the R1 subunit from ribonucleotide reductase but no R2 ORF is present. Detailed analysis of the U28 amino acid sequence indicated that a number of essential R1 catalytic residues are absent. Cloning and expression of the U28 protein in E. coli and its subsequent characterization in subunit interaction and enzyme activity assays confirmed that it is not a functional equivalent of a herpesvirus R1. In the absence of the R2 gene, we propose that the R1 ORF has evolved a distinct, as yet unidentified, function not only in human herpesvirus-7 but also in other human betaherpesviruses.

Ribonucleotide reductase (RR) plays an essential role in the de novo synthesis of DNA in all living organisms by catalysing the conversion of all four ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates. Currently, three distinct classes of RR enzymes have been identified that differ in their primary structure and in their means of radical generation (for reviews see Reichard, 1993; Stubbe & Riggs-Gelasco, 1998; Fontecave, 1998). A free radical is an essential component for the chemically difficult replacement of the ribose 2′-OH in ribonucleotides with hydrogen in deoxy-ribonucleotides (Logan et al., 1999). Class II RR enzymes are mono- or homodimeric proteins that use the cofactor adenosylcobalamin for transient radical generation (Licht et al., 1996). Class III enzymes are heterodimeric proteins that function under anaerobic conditions and generate a glycy radical using an S-adenosylmethionine cofactor (Logan et al., 1999). Class I RRs, for which the E. coli enzyme serves as a prototype, are also composed of two heterodimeric proteins, termed R1 and R2.

Protein R2 provides a stable tyrosyl radical, associated with a dinuclear iron centre that contributes to the catalytic process by long-distance hydrogen bond-mediated radical transfer to the active site in R1 (Ekberg et al., 1998). The mammalian cell and the herpesvirus RRs are Class I enzymes. Proteins R1 and R2 from many herpesviruses have been identified and the herpes simplex virus-1 (HSV-1) enzyme has been characterized most fully (for review see Conner et al., 1994). HSV-1 RR is a target for antiviral chemotherapy and has provided a paradigm to study peptides that disrupt protein-protein interactions as a route for antiviral drug development (Liuzzi et al., 1994).

Amongst the alpha- and gammaherpesviruses, the R1 and R2 subunits are highly conserved and key catalytic residues are readily identifiable (Willoughby et al., 1997). In protein R1 certain residues are absolutely conserved including five catalytically active cysteines, three that are grouped in the active site and two that are located at the C terminus, and a pair of tyrosines, involved in electron transfer from the radical centre in R2 to the R1 active site cysteines (Ekberg et al., 1996).

DNA sequencing of the human betaherpesviruses, human cytomegalovirus (HCMV), human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7) has identified an ORF with homology to R1 (Chee et al., 1990; Gompels et al., 1995; Nicholas, 1996; Megaw et al., 1998) but an ORF for R2 is absent, suggesting that these viruses do not express a functional RR enzyme. We report here on the cloning, expression and characterization of the proposed R1 protein (U28) from HHV-7 and our results demonstrate that the polypeptide is not a functional R1 protein.

Submission of the U28 amino acid sequence to database searches identifies the protein as an R1 homologue with the closest matches occurring amongst other herpesvirus R1 subunits. Initially therefore, we aligned the amino acid sequence of U28 with that of the equine herpesvirus-4 (EHV-4) R1, chosen as a representative of herpesvirus R1 proteins (Fig. 1). Although a 22% amino acid identity exists between the two sequences the alignment demonstrates that essential catalytic residues are absent from the HHV-7 polypeptide. Significantly, only one of the five essential cysteines (EHV-4 amino acid 223) is conserved with the remaining four (EHV-4 amino acids 452, 462, 784 and 787) absent from the U28 sequence. The proposed GxGxxG nucleotide-binding site
Fig. 1. For legend see facing page.
Fig. 1. Amino acid sequence alignment of EHV-4 R1 (top) with HHV-7 U28 (bottom). The highly conserved EHV-4 R1 residues that are discussed in the text and have been shown to have a direct catalytic role in *E. coli* RR are cysteines 223, 452, 462, 784, 787, tyrosines 764, 765 (all highlighted and in bold) and a GxGxxG motif at residues 517–522 (highlighted only). Pro-743 and Ala-744, equivalent to Pro-1090 and Ala-1091 in HSV-1 R1 and directly involved in subunit interaction, are in bold and underlined. EHV-4 R1 was chosen as a representative of the herpesvirus R1 subunits rather than HSV-1 R1, which has an additional 300 amino acids that form a unique N-terminal extension not required for ribonucleotide reduction (Conner *et al.*, 1993).

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Plasmid pED594.5, containing almost the entire U28 ORF except for the first methionine codon, was kindly provided by John Nicholas, Johns Hopkins Oncology Center, Baltimore, MD, USA. The U28 DNA was excised from this plasmid by digestion with *Bam*HI and *Xho*I restriction enzymes and ligated into the expression plasmid pET28b, digested with the same enzymes. Following transformation of *E. coli* and kanamycin selection, positive clones were grown overnight and were used to prepare both plasmid and glycerol stocks. Sequencing of plasmids from several positive clones confirmed the presence of the U28 ORF with all 1500 bases obtained exactly matching the published sequence (data not shown). Polypeptide expression from pET28b incorporates a T7-tag at the N terminus of the protein allowing its detection in Western blotting with a T7-tag monoclonal antibody (Cambridge Bioscience). U28 protein expression was induced by addition of 150 mM IPTG to 300 ml cultures and 35% ammonium sulphate fractions were prepared from the harvested cultures as described in Conner *et al.* (1993). A 93 kDa, T7-tagged polypeptide was detected by Western blotting in the 35% ammonium sulphate fraction, consistent with the 805 amino acids encoded by the U28 DNA and the additional N-terminal, T7-tag residues (data not shown but see Fig. 2, lane 5). Protein U28 was purified from the 35% ammonium sulphate fraction using the T7-tag monoclonal antibody immobilized on an immunoaffinity column (Fig. 2). For purification, the ammonium sulphate fraction from a 300 ml culture (Fig. 2, lane 1) was suspended in 2 ml PBS, pH 7.3, with 0.1% Tween 20 and applied to a 1 ml T7-tag antibody affinity column equilibrated in the same buffer. One ml fractions were collected and after unbound material was eluted (lanes 2 and 3) the column was washed extensively with 20 ml PBS. Purified U28 was then eluted using 5 ml 0.1 M citric acid, pH 2.2 (lanes 4 and 5); the low pH was neutralized immediately by 150 µl 2 M Tris solution, pH 10.4, present in the collection tubes. One hundred-and-twenty µg of purified protein was obtained from 300 ml of bacterial culture.

Protein U28 was analysed for its ability to interact with purified protein R2 from both HSV-1 and EHV-4 in a subunit interaction ELISA (Conner *et al.*, 1993). Bacterially expressed HSV-1 R1 and R2 were purified as described by Conner *et al.* (1993) and Lankinen *et al.* (1991) respectively. Cloning,
Fig. 2. Coomassie-stained SDS–polyacrylamide gel demonstrating purification of U28 protein following its cloning and expression in E. coli. Lane 1 shows the bacterial extract that was applied to the T7-tag immunoaffinity column, lanes 2 and 3 show the unbound material and lanes 4 and 5 show the bound material eluted by the low pH buffer. Lane M shows molecular mass markers (sizes are indicated on the right-hand side of the gel).

Fig. 3. Charts demonstrating that U28 protein cannot interact with other herpesvirus R2 proteins to form an active enzyme. All assays were performed in duplicate and mean values are shown. (A) Results from the subunit interaction ELISA showing effects on OD630 of increasing amounts of HSV-1 R1 (●), EHV-4 R1 (▲) and U28 (○) added to EHV-4 R2-coated wells. (B) Results from enzyme activity assays showing [3H]CTP incorporation into DNA. Assays were performed with 40 µl of 35% ammonium sulphate fractions containing either EHV-4 R1 (E-R1), HSV-1 R1 (H-R1) or U28 alone or in the presence of 2 µg R2. HSV-1 R1 was assayed with HSV-1 R2 (H-R1 + H-R2), EHV-4 R1 was assayed with EHV-4 R2 (E-R1 + E-R2), EHV-4 R1 was assayed with HSV-1 R2 (E-R1 + H-R2) and U28 was assayed with both HSV-1 (U28 + H-R2) and EHV-4 R2 (U28 + E-R2).

expression and purification of EHV-4 R1 and R2 will be described in detail elsewhere. Briefly, EHV-4 R1 and R2 DNAs were PCR-amplified separately from the viral genome, ligated into pET28a expression vectors and expressed in E. coli following IPTG induction. EHV-4 R2 purification was achieved using the HSV-1 R2 method (Lankinen et al., 1991) and EHV-4 R1 was purified using the T7-tag immunoaffinity protocol as described for the U28 protein. For the R2 interaction ELISA, microtitre plates were coated with 0.3 µg purified protein. After blocking with 5% milk powder, plates were incubated for 1 h with increasing amounts of HSV-1 R1, EHV-4 R1 and U28 present in 35% ammonium sulphate fractions. Interactions of HSV-1 R1, detected using polyclonal antibody 106 (Conner et al., 1993), and EHV-4 R1, detected with the T7-tag monoclonal antibody, with EHV-4 R2 are shown in Fig. 3(A). Interactions of HSV-1 and EHV-4 R1 with HSV-1 R2 were also detected (data not shown). Significantly, no interaction of U28, probed with the T7-tag monoclonal antibody, either with HSV-1 (data not shown) or EHV-4 (Fig. 3A) R2 was observed.

U28 was also tested for its ability to complement both HSV-1 and EHV-4 R2 in an RR assay (Jong et al., 1998). This assay system couples ribonucleotide reduction with a DNA polymerase reaction and measures the incorporation of [3H]dCTP, converted from the [3H]CDP substrate by the
combined action of ribonucleotide reductase and a nucleotide diphosphate kinase present in the 35% ammonium sulphate fractions used in the assay as a source of R1 proteins, into newly synthesized DNA. Increasing amounts of the 35% ammonium sulphate fraction containing protein U28 were incubated with either 2 µg purified HSV-1 or EHV-4 R2 and RR assays were performed. No radiolabelled DNA was detected in any of these assays (Fig. 3B). Radiolabelled DNA was detected when similar assays were performed with HSV-1 R1/R2, EHV-4 R1/R2 and with the hetero-subunit complex formed by EHV-4 R1/HSV-1 R2 (Fig. 3B). Importantly, [³²P]dCTP was only incorporated into DNA when active R1 and R2 subunits were present (Fig. 3B). Although the level of activity detected with the EHV-4 R1/HSV-1 R2 enzyme was lower than that observed with the two wild-type enzymes, it was at least 10-fold greater than background levels.

Our results clearly demonstrate that the U28 protein is not the functional equivalent of the R1 subunit of herpesvirus RR. The cloned and bacterially expressed protein was unable to complex with the R2 subunits from HSV-1 and EHV-4 in both RR activity and subunit interaction assays. The sites of subunit interaction, particularly at the R2 C terminus, are well-conserved amongst alpha- and gammaherpesviruses and in our assay system both HSV-1 and EHV-4 R1 were able to form hetero-subunit complexes with EHV-4 and HSV-1 R2 (see Fig. 3A). Importantly, any interaction of U28 with a R2 subunit, including the remote possibility of interaction with the host cell R2, would not result in a functional RR as most of the key catalytic residues essential for R1 activity are absent from U28. It is highly improbable also that U28 forms a single subunit RR as other single subunit enzymes are restricted to bacteria and archaea and we could detect no RR activity with U28 in our assay system. However, interaction with other mammalian cell proteins (known or unknown) to form an active RR complex cannot be completely ruled out.

We analysed U28 of HHV-7 as a representative of the R1 homologues identified also in HCMV and HHV-6 and we conclude that betaherpesviruses have no requirements for RR activity. The absence of this key nucleotide metabolizing enzyme is most probably a consequence of the biological properties of this group of viruses: in particular, their restricted host ranges and long replication cycles. We propose that during the evolution of the betaherpesvirus subfamily there was minimal requirement for a virus-encoded RR and, consequently, the R2 ORF was lost, allowing the R1 ORF to mutate and develop another, as yet unidentified, function. Interestingly, HSV-1 R1 possess a unique N-terminal extension of 300 amino acids that plays an additional role in virus replication (Conner et al., 1995; Smith et al., 1998) and the function of the U28 protein may be related to this. The availability of the bacterially expressed and purified U28 will allow its further characterization and the development of specific antibody reagents to address more fully the function of this protein during HHV-7 infection.

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References


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