Identification of the UL4 protein of herpes simplex virus type 1

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Herpes simplex virus type 1 gene UL4 is predicted to encode a 199-amino-acid protein with a molecular mass of 21.5 kDa. We report here identification of this protein and its localization in the nuclei of infected cells. Antisera raised against oligopeptides corresponding to the C terminus of the predicted UL4 protein were used for identification of a 25 kDa protein as the product of the UL4 gene. This protein was not detected in cells infected with a UL4 defective mutant virus, but was synthesized by coupled in vitro transcription–translation of the UL4 gene. Synthesis of the 25 kDa protein was blocked by phosphonoacetic acid, an inhibitor of DNA synthesis, indicating that the UL4 gene is expressed with γ kinetics. Subcellular fractionation showed the protein to be localized in the nucleus. It was not detected in virions or light particles.

The genome of herpes simplex virus type 1 (HSV-1) has been completely sequenced (McGeoch et al., 1985, 1986, 1988) and shown to contain at least 70 open reading frames (ORFs) potentially encoding proteins. For many of these ORFs the gene products are known, and their functions and regulation of expression have been studied in detail. For other genes the products have not yet been identified nor have functions been assigned to them. One such gene is UL4, which is predicted to encode a 199-amino-acid protein with a molecular mass of 21.5 kDa (McGeoch et al., 1988). Analysis of a mutant from which a part of the UL4 gene was deleted suggests that the UL4 gene product is not essential for replication of virus in cell culture (Baines & Roizman, 1991).

Homologues of the UL4 gene are present in other members of the Alphaherpesvirinae, specifically the HSV-2 gene UL4 (McGeoch et al., 1991), varicella-zoster virus gene 56 (Davison & Scott, 1986), equine herpesvirus-1 gene 58 (Telford et al., 1992), pseudorabies virus gene UL4 (Dean & Cheung, 1994) and bovine herpesvirus-1 gene UL4 (Vlek et al., 1995). Conservation of the gene suggests that it plays a role in the virus life-cycle.

Three different methods were used to identify the HSV-1 UL4 gene product. First, extract from cells infected with wild-type virus was analysed using UL4-specific antisera. Proteins from uninfected BHK cells (Macpherson & Stoker, 1962) or cells infected with HSV-1 17syn+ (Brown et al., 1973) were separated by SDS–PAGE and then blotted onto ECL nitrocellulose membranes. The filters were stored overnight at 4 °C in modified 5× Denhardt’s solution (Cohen et al., 1984) before incubation with antiserum. Antiserum specific for UL4 were raised in rabbits against multiple antigenic peptides (MAPs) (Tam, 1988) as described previously (McLean et al., 1990), except that 300 µg was used per immunization per rabbit. Antiserum 62 and 98 were raised against peptides (IQLSPTEYADKLGLS)K, (QLSPTEYADKLGLS)K, (QLSPTEYADKLGLS)K, (QLSPTEYADKLGLS)K, respectively. These peptides correspond to the C terminus of the predicted UL4 protein of HSV-1 strains 17syn+ and KOS, respectively, and differ only in an alanine to threonine substitution at amino acid 189. Antiserum 62 recognized three proteins in infected cells that were not detected in mock-infected cells (Fig. 1A) and were not recognized by preimmune serum (data not shown). These bands are marked with an arrowhead and circles. The lower band (arrowhead) migrated with an apparent molecular mass of 25 kDa, which is slightly larger than the predicted size of the UL4 protein (21.5 kDa). The apparent molecular masses of the other two bands are 47 and 74 kDa. Incubation of antiserum 62 with 10 µg/ml of oligopeptide (Fig. 1B) or 1 µg/ml (data not shown) prior to Western blotting consistently blocked the reaction with the smallest band only, suggesting that the antiserum was specific for the 25 kDa protein.

The UL4 protein band was rather weak in many experiments and sometimes almost undetectable. Weak signals were observed both by immunoprecipitation and Western blot in...
Fig. 1. Detection of the UL4 protein in extracts from infected cells and by coupled in vitro transcription–translation from the UL4 gene. (A) BHK cells were infected with strain 17syn + and cell extracts prepared. Proteins from uninfected (M) or infected (I) cells were separated on an SDS–polyacrylamide gel (5–12.5% gradient) and blotted onto an ECL nitrocellulose filter which was then incubated with antiserum 62. The positions of molecular mass markers (Bio-Rad) are shown. The band at the expected position of the UL4 protein is labelled with an arrowhead. Additional bands reacting with the immune serum are labelled (D). A cellular protein slightly below the position of the UL4 protein is marked (E). (B) Extracts from cells infected with strain 17syn + were subjected to SDS–PAGE (9% polyacrylamide) and blotted onto ECL nitrocellulose filters which were individually incubated with immune serum 62 which had been preincubated with UL4 17syn + oligopeptide at the concentrations indicated. Relevant portions of the filters are shown. Molecular mass markers used were as in (A). (C) The UL4 gene was inserted into plasmid pCDNA3, coupled transcription–translation performed, the proteins labelled with [35S]methionine and separated by SDS–PAGE as in (B). 14C-labelled molecular mass markers (Amersham) (lane 4) and reaction mixtures without DNA (lane 1), with the luciferase gene (lane 2) and with the UL4 gene (lane 3), respectively, were run in separate lanes. The gel was infused with PPO and then dried and exposed to X-Omat AR5 film. The band corresponding to the UL4 gene product is indicated with an arrowhead.

As a second approach to identifying the UL4 gene product(s), the UL4 gene was cloned into an expression vector and coupled in vitro transcription–translation was performed. Plasmid pTE1 (kindly provided by Alasdair MacLean, University of Glasgow, UK) containing the complete UL3 and UL4 genes and part of the UL5 gene of HSV-1 17syn + was cut with BamHI and HindIII, and a fragment containing the complete UL4 gene and the 3′ portion of the UL5 gene was inserted into pGEM-7Zf(®) (Promega). A SacI–HindIII fragment containing the same UL4 and UL5 sequences and a preserved BamHI site was incubated with Tru9I, which cuts immediately downstream of the UL5 gene. Ends were made blunt using Klenow enzyme, the fragment cut with BamHI and then inserted into the Smal and BamHI sites of pCDNA3.
(Invitrogen) to generate the UL4 expression plasmid used for coupled in vitro transcription–translation in the TnT T7 reticulocyte lysate system (Promega). Proteins were radio-
labelled with [35S]methionine, separated by SDS–PAGE and the gel infused with PPO (Bonner & Laskey, 1974) before drying and autoradiography. Fig. 1(C) shows that a protein of molecular mass 25 kDa (arrowhead) was made from vector DNA, but not from irrelevant DNA (encoding luciferase) or by endogenous activity in the reaction mixture. The higher molecular mass proteins (47 and 74 kDa) were not detected. This suggests that the 25 kDa species is the product of the UL4 gene. The protein seems to form two adjacent bands in the gel shown in Fig. 1(C). Although this could be consistent with post-translational modification occurring in the in vitro system, this possibility has not been analysed further.

The third approach for identification of the UL4 protein was analysis of the proteins made by a UL4 defective mutant. A UL4 mutant virus that encodes only the first 40 amino acids of the UL4 protein was made by cosmid recombination (Cunningham & Davison, 1993) from HSV-1 17syn+ by inserting the oligonucleotide GATCTAATCTAGATTA into the Smal site at residue 12305 in the HSV-1 genome (McGeoch et al., 1988). This oligonucleotide contained stop codons in all three reading frames. Extracts from cells infected with the mutant or with the wild-type viruses 17syn+ and KOS were analysed by Western blots using the UL4-specific antiserum. The antibodies were concentrated by resuspending the material from ten 35 mm dishes in 500 µl PBS containing 0.2 mM PMSF. Furthermore, the AMDEX conjugate was used. The right panel in Fig. 2(A) shows that the only difference between the UL4 mutant and its parental strain (17syn+) is the absence of the 25 kDa band (arrowhead). This band was also not detected using the KOS antiserum (left panel). Similar observations were made using another UL4 mutant from strain KOS and its revertant (Jun et al., 1998). Fig. 2(A) also shows that the 17syn+ antiserum recognized the UL4 protein from strain 17syn+ but not from KOS. The KOS antiserum, on the other hand, reacted with the proteins encoded by both strains 17syn+ and KOS. It should be noted that concentrated antigens and the AMDEX system used increased the background so that several protein bands are observed, but only the 25 kDa band is not seen in extracts of cells infected with the UL4 mutant.

The presence of the 47 and 74 kDa proteins in cells infected with the UL4 mutant provides additional indication of the unrelatedness of these proteins to UL4. One could envisage the possibility that the 47 and 74 kDa proteins were derived from larger transcripts coterminal with that of UL4, since the antiserum was raised against an oligopeptide corresponding to the C terminus of the predicted UL4 protein. This possibility is unlikely because the proteins were detected in cells infected with the virus mutant.

To analyse the subcellular localization of the UL4 protein, concentrated cytoplasmic and nuclear fractions were prepared by two different methods. Lactate dehydrogenase (LDH) activity and monoclonal antibody Z1F11 recognizing the UL42 protein (Murphy et al., 1989) were used as cytoplasmic and nuclear (Goodrich et al., 1989) markers, respectively. The nuclei appeared intact after treatment with a Dounce homo-
genizer (Dignam et al., 1983; Sternsdorf et al., 1997) (Fig. 2 B, upper panel), but traces of cytoplasm were occasionally observed (data not shown). After sequential incubation with two detergents in a homogenization buffer (Birckbichler & Pryme, 1973) (lacking heparin), LDH was present exclusively in the cytoplasm, but some nuclear leakage occurred (data not shown). Fig. 2(B) shows that, independent of the method of preparation, the UL4 protein (marked ‘△’) was present in the nuclear fraction from cells infected with wild-type but not with UL4 mutant virus. A very weak UL4 band was occasionally observed in the cytoplasm from cells infected with strain 17syn+. The mutant and wild-type viruses were also used to examine the possibility that the UL4 protein is structural. Purified virions and light particles were prepared from either wild-type virus or the UL4 mutant as described by Szilágyi & Cunningham (1991) with slight modifications (Sathananthan et al., 1996). Again, concentrated antigens were used. Virions from three roller-bottles were pelleted at 60,000 r.p.m. and 4 °C for 40 min in TLA 120.2 rotor in a Beckman TLX ultracentrifuge and resuspended in a final volume of 50 µl PBS containing 0.2 mM PMSF. Light particles were pelleted at 80,000 r.p.m. for 4 h and resuspended in a final volume of 90 µl. The preparations were stored at −80 °C until further analysis by Western blot using the AMDEX conjugate. The results are shown in Fig. 2(C). The UL4 protein was readily detected in the cell extract (arrowhead), but not in virions or in light particles. As a control, antiserum LA2-3, specific for the VP16 tegument protein (kindly provided by Steven J. Triezenberg, Michigan State University, USA), reacted with a protein in all extracts except for that from uninfected cells. We conclude that the UL4 protein is either non-structural or present in the virion in amounts below the present limit of detection. A 47 kDa protein was detected in virions and light particles from wild-type virus as well as from the UL4 mutant and thus is not expressed from gene UL4. Similarly, a prominent 115 kDa band (marked ‘x’ in Fig. 2C) reacting with the UL4 antibody was present in both 17syn+ and UL4 mutant virion preparations, suggesting that it also is not a product of the UL4 gene. The origins of these proteins are unknown and have not been investigated further.

The kinetics of induction of the UL4 protein were then examined. As shown in Fig. 3 the protein (arrowhead) was detected as a minor band as early as 2 h after infection and was markedly upregulated at 8 h post-infection. In the presence of phosphonoacetic acid (PAA) it was not detected at any time-point after 4 h. Since exposures using the ECL system may vary slightly from one filter to another, extracts from PAA-treated and untreated cells were run side-by-side in the same gel, but the UL4 protein was still undetectable in the first set of
Fig. 2. Identification of the UL4 protein in nuclei of infected cells, but not in virions or light particles. (A) Extracts from uninfected cells or from cells infected with strain 17syn⁺, strain KOS or the UL4 defective mutant were subjected to SDS–PAGE (12.5% polyacrylamide gels), proteins blotted onto ECL nitrocellulose filters, and the filters incubated with immune serum 98 (KOS Ab) and 62 (17⁺ Ab), respectively. The filters were then incubated with the AMDEX conjugate. The positions of low-range molecular mass markers (Bio-Rad) are indicated. A protein present after infection with wild-type virus only is marked with an arrowhead. The positions of the 47 kDa and 74 kDa bands reacting with the immune sera are indicated ( ). (B) Uninfected cells and cells infected with strain 17syn⁺ or with the UL4 mutant virus were subjected to subcellular fractionation using either a Dounce homogenizer (pestle B) (upper two panels) or sequential incubation with 0.1% Nonidet P-40 and 0.1% sodium deoxycholate (lower panel). The final volume of each fraction from 10⁷ cells was 200 to 400 µl. Nuclei prepared by detergent treatment were pelleted at 3000 r.p.m. and 4 °C for 10 min in a Kubota 8700 centrifuge and washed twice. Proteins were separated by SDS–PAGE and blotted as in (A), and the filters incubated with either MAb Z1F11 (upper panel, UL42 Ab) or with immune serum 62 (lower two panels, UL4 Ab). Reacting antibodies from mouse or rabbit were detected by anti-mouse IgG coupled to horseradish peroxidase (rabbit anti mouse IgG–HRP, DAKO P0161) and with the AMDEX conjugate, respectively. Only the relevant portions of the filters are shown. A protein present in the nuclear fraction from cells infected with strain 17syn⁺ is marked ( ). (The fuzzy spot in the left lane in the middle panel is an artifact.) (C) Extracts (Extr.) were prepared from either uninfected cells or from cells infected with strain 17syn⁺. Virions (H) and light particles (L) from 17syn⁺ and from the UL4 defective mutant were purified in Ficoll gradients. Proteins were separated by SDS–PAGE and blotted as in (A), the filter incubated with immune serum 98 (UL4 Ab) and then with the AMDEX conjugate. Another filter containing the same material from an identical gel was incubated with the VP16 antiserum (VP16 Ab) and then with the AMDEX conjugate. The UL4-specific band in extract from infected cells is marked with an arrowhead. The positions of other proteins reacting with the UL4 antisera are indicated with circles. A prominent high molecular mass band observed in virions only is labelled (x).
experiments (data not shown). At 4 h post-infection (+PAA) a very weak band was occasionally observed (Fig. 3). These results suggest that the UL4 product belongs to the γ group of proteins. The 47 and 74 kDa bands (circled) are present in large amounts 2 to 4 h post-infection independent of the effects of PAA, consistent with the previous conclusion that these proteins are not related to the UL4 gene product. Taken together, the data show that the HSV-1 UL4 gene encodes a 25 kDa late nuclear protein which was not detectable in the virions or light particles.

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References


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