Transfer of UL41, the gene controlling virion-associated host cell shutoff, between different strains of herpes simplex virus

M. L. Fenwick and R. D. Everett

Studies with mutant viruses have suggested that the product of gene UL41 of herpes simplex virus type 1 (HSV-1) controls the virion-mediated inhibition of cellular protein synthesis as well as the rate of degradation of viral mRNAs. HSV-1 strain 17+ has a weak host shutoff function, whereas HSV-2 strain G shuts off strongly. A gene of HSV-2(G), judged from its position in the genome to be the probable analogue of gene UL41 of HSV-1, was inserted into the non-essential thymidine kinase gene of HSV-1(17+). The recombinant virus, 17G41, exhibited a strong shutoff function and its immediate early mRNA did not accumulate in the presence of cycloheximide. It resembled HSV-2(G) in these respects and not the parent, confirming the function of the transferred gene. Recombinant virus 17G41 carries the UL41 genes of both strains, 17+ and G, and in this situation the strong shutoff function was dominant. However, after mixed infection with equal multiplicities of 17G41 and HSV-1(17+) the weak shutoff function was dominant. The recombinant, 17G41, was further modified by insertion of a lacZ expression cassette into the coding region of the original gene UL41 (17+). The resulting virus, 17(41-)G41, also had a strong shutoff activity but grew poorly in tissue culture.

Introduction

Some strains of herpes simplex virus (HSV) quickly suppress the synthesis of the host cell proteins while proceeding to make their own. This early shutoff is caused by a component of the virion (Nishioka & Silverstein, 1978; Fenwick & Walker, 1978) and is accompanied by degradation of cellular mRNA (Fenwick & McMenamin, 1984; Mayman & Nishioka, 1985; Schek & Bachenheimer, 1985). Studies with intertypic recombinant viruses and shutoff-defective mutants have shown that the effect is controlled by the vhs gene approximately at position 0-6 on the HSV-1 genome map (Morse et al., 1978; Kwong et al., 1988). On the basis of this mapping, the vhs gene corresponds to gene UL41 (McGeoch et al., 1988). Inactivation of the vhs gene was also associated with increased stability of viral mRNAs (Read & Frenkel, 1983; Oroskar & Read, 1987, 1989; Kwong & Frenkel, 1987; Kwong et al., 1988). Other experiments have suggested the existence of a counter-acting viral function which protects viral immediate early (IE) mRNA from degradation and can be trans-dominant in mixed infection (Fenwick & Clark, 1983). HSV-1(17+) and HSV-2(G) are two strains of virus which differ markedly in both virion-associated shutoff activity and in IE mRNA stability. HSV-1(17+) causes only slight inhibition, and its IE mRNA accumulates in the presence of cycloheximide (CX) added at the time of infection and is translated on removal of the inhibitor. In contrast, HSV-2(G) shuts off host synthesis strongly; also its IE mRNA is very unstable and does not accumulate unless protein synthesis is allowed (Fenwick & Clark, 1983; Fenwick & Owen, 1988). To investigate the functions of gene UL41 and in particular the question of whether it alone determines both phenotypes, we have isolated the analogous gene from HSV-2(G) and inserted it into the non-essential thymidine kinase (TK) gene of HSV-1(17+). The recombinant virus, 17G41, containing the UL41 genes of both 17+ and G, possessed both the strong shutoff and the viral mRNA instability characteristic of HSV-2(G). Apparently the UL41 gene of G was dominant in the diploid situation and controlled both functions.

In contrast, after simultaneous mixed infection, HSV-1(17+) prevented the shutoff by recombinant 17G41. This observation is consistent with the report (Hill et al., 1985) that in cells infected with two strains of virus, the one that did not suppress host protein synthesis competitively inhibited shutoff by the other. These results are discussed in terms of the possible mode of action of the UL41 gene product.

The recombinant virus 17G41 was modified further to
inactivate its original UL41 gene by insertion of a lacZ gene. The resulting virus, 17(41-)G41, still had strong shutoff activity but did not grow to a high titre in tissue culture.

**Methods**

**Viruses and cells.** The viruses used were HSV-1 Glasgow strain 17* (Brown et al., 1973), HSV-2 strain (G) (Ejercito et al., 1968), obtained from Dr B. Roizman, and HSV-2 strain HG52 (Timbury, 1971). They were grown in BHK C13 cells in Glasgow modified Eagle's medium supplemented with 10% newborn calf serum.

**Plasmids.** Plasmid pTK1 (containing the 3.6 kb BamHI p fragment of HSV-1(17*) DNA in pAT153) has been described previously (Wilkie et al., 1979). Plasmid pBamt, containing the BamHI t fragment of HSV-2(2G) in pAT153, was kindly provided by Dr J. MacLaughlan. It was used as a probe to detect clones bearing the Bgl II n fragment of HSV-2 (G). Plasmid pGIX80 comprises the HindIII fragment of HSV-1(17*) in pAT153 and was constructed by Dr B. Matz at the Institute of Virology, Glasgow. It contains all or part of genes UL41 to UL46. Plasmid pFJ3 contains the lacZ gene linked to the simian virus 40 (SV40) early promoter in a 4-1 kb cassette flanked by XbaI sites and was kindly provided by Dr F. J. Rixon. All other plasmids were constructed in the course of this work, using standard recombinant DNA techniques as outlined in the text.

**Transfection with viral DNA and isolation of recombinant viruses.** Viral and plasmid DNAs were cotransfected into BHK cells in 35 mm dishes by the method of Stow & Wilkie (1976). After c. p.e. became evident, the cells, resuspended in their growth medium, were disrupted by ultrasonication and the progeny virus was titrated on BHK cells with an agarose overlay. Single plaques were picked and used to infect BHK cultures in the 10 mm wells of a tissue culture tray (Linbro). The medium containing cell-released virus was stored at −70 °C. Total cellular DNA was prepared from infected cells (Stow et al., 1983) and analysed by restriction enzyme digestion, Southern blotting and hybridization with nick-translated probes (Southern, 1975; Rigby et al., 1977). Isolates with the desired restriction patterns were further plaque-purified two more times before large scale stocks were prepared.

Recombinant virus 17G41, which contains an insertion in the TK gene, was selected from the progeny of the initial transfection by growth and plaque purification in medium containing bromodeoxy- cytidine (BcdR) (100 μg/ml), in which only TK-deficient viruses can grow (Stow et al., 1978). Virus 17(41-)G41 was detected in the transfection progeny and during subsequent purification by the inclusion of X-Gal (Sigma) in the overlay at a final concentration of 200 μg/ml. Viruses bearing an inserted lacZ gene formed blue plaques (F. J. Rixon, personal communication).

**Assay of host cell shutoff and IE RNA stability.** BHK cells were infected during incubation for 20 min at room temperature with a multiplicity of 10 p.f.u./cell. At zero time the inoculum was removed and the incubation continued at 37 °C in the presence of actinomycin (2 μg/ml) or CX (50 μg/ml). To measure shutoff, actinomycin-treated cells were labelled with 3H)methionine or 14C-labelled amino acids between 2 and 3 h post-infection (p.i.). To assess the accumulation of IE mRNA, CX-treated cells were washed at 3 h p.i. and labelled for 1 h in the presence of actinomycin (2 μg/ml). Stability of IE RNA was measured by adding actinomycin to duplicate CX-treated cultures at 3 h p.i. and then by washing and labelling proteins from 4 to 5 h p.i.

**Results**

**Insertion of the UL41 gene of HSV-2(G) into pTK1**

The Bgl II n fragment of HSV-2 DNA spans the region between 0-58 and 0-63 map units. It contains a gene encoding a 61K protein (Galloway et al., 1984) which, on the basis of its position in the genome and on other criteria, we thought to be the probable analogue of gene UL41 of HSV-1 (McGeoch et al., 1988). HSV-2(G) DNA was digested with Bgl II and the fragments were ligated into the BamH I site of pUC9. The desired clone (pGBl II n) was detected by colony hybridization with a 32P-labelled probe containing the BamH I t fragment of HSV-2(HG52) DNA.

According to the restriction mapping of Galloway et al. (1984), the putative shutoff gene (UL41 equivalent) is located within two contiguous SstI fragments in the Bgl II n sequence. This region was isolated by partial digestion of pGBgI In with SstI and elution of the required 3.6 kb fragment from a gel. It was then ligated into the unique SstI site in the TK gene in pTK1. The resulting plasmid was called pMF1 (Fig. 1).

**Construction of recombinant virus 17G41**

To transfer the UL41 gene of strain G to 17*, pMF1 was cotransfected with HSV-1(17*) DNA into BHK cells. In order to increase recombination frequency, pMF1 was first digested with EcoRI, generating a linear fragment containing the TK coding region of HSV-1(17*) with the inserted UL41 gene of HSV-2(G). Viruses unable to express TK were selected from the resulting mixed progeny by growth in the presence of BcdR as described in Methods. The DNAs extracted from 40 isolated plaques were digested with BamH I and probed with 32P-labelled pTK1 after Southern blotting. Four samples yielded radioactive bands of the sizes expected (2-8 and 4-4 kb) from the insertion of the required 3-6 kb UL41-containing fragment with its single BamH I site (Fig. 1). The corresponding cell-released viruses were plaque-purified further in the presence of BcdR. Seven cell-associated viral DNAs were obtained and again checked by Southern blotting. One of these, represented by lane 4 in Fig. 2, was selected and the corresponding cell-released virus was designated 17G41. It was titrated and used to prepare a virus stock by low multiplicity passage in BHK cells. The faint band at 3-6 kb in Fig. 2, lane 4, may represent some rearrangement between the HSV types 1 and 2 UL41 genes or possibly a slight
Transfer of HSV UL41 gene

Fig. 1. Construction of pMF1. The fragment obtained by partial digestion of the BglII n fragment of HSV-2 with SstI (lower line), was inserted in the orientation shown at the SstI site of the BamHI p fragment of HSV-1(17+) (top line), contained in pTK1. The numbers indicate sizes of restriction fragments (kbp).

Fig. 2. Detection of an insert containing a BamHI site in the BamHI p region of HSV-1(17+) DNA. The Southern blot of BamHI-digested DNAs was probed with 32P-labelled pTK1. Lanes 1 to 7, viral DNAs (lanes 3, 5 and 7 contained insufficient DNA). Lanes 9 and 10 represent pMFI and pTK1 digested with BamHI. The middle band in lane 9 indicates vector DNA (3.7 kb).

contamination with wild-type virus DNA. It was barely detectable in the virus stock used for later experiments and was not present in subsequent isolates, such as 17(41-)G41 (Fig. 6, lane 5).

Polypeptides expressed by recombinant virus 17G41

BHk cells were mock-infected or infected with HSV-1(17+) or with recombinant virus 17G41 at a multiplicity of 10 pfu per cell. Proteins were labelled with [35S]methionine from 3.5 to 4.5 h p.i. and examined by electrophoresis and autoradiography (Fig. 3). The patterns were similar except for the absence from lane 3 (17G41-encoded proteins) of a band at about the 41K position, presumed to represent TK, and the enhancement of a smaller polypeptide, possibly an incomplete or hybrid TK form.

17G41 has strong shutoff activity

The ability of 17G41 to suppress cellular protein synthesis in the absence of viral gene expression was examined by infecting in the presence of actinomycin before labelling. As can be seen in Fig. 4(a) HSV-1(17+) (lane 2) causes partial suppression but the introduction of the equivalent UL41 gene from strain G into 17+ has resulted in a virus with a strong virion-associated shutoff activity (Fig. 4a, lane 3).

The IE mRNA of 17G41 is unstable

The accumulation and stability of the viral IE mRNA in the absence of viral gene expression was assessed by measuring the rate of protein synthesis after reversal of a CX-induced block. Cells were incubated with CX from the time of infection with strain 17+ or with recombinant virus 17G41. At 3 h p.i. the CX was removed by washing and proteins were labelled with [35S]methionine (Fig. 4a, lanes 5 to 7). In cells infected with 17+ the IE
polypeptides Vmw175 and Vmw110 were produced in large amounts, together with many host proteins (lane 6). In contrast, in cells infected with 17G41, the synthesis of both host and viral IE proteins was much reduced (lane 7). In addition, the IE mRNA of 17+ was stable (as measured by labelling with [35S]methionine following a 1 h chase period in the presence of actinomycin before removal of CX), whereas that of 17G41 was no longer detectable (Fig. 4b). Thus 17G41 resembles HSV-2(G) (Fenwick & Clark, 1983) both in its strong host shutoff activity and in the instability of its IE RNA.

Gene UL41 of HSV-1(17+) is dominant in mixed infections

The results presented above showed that in the recombinant virus 17G41 containing two UL41 genes the function of the newly introduced gene of G was dominant. This appeared to conflict with the results of previous experiments with mixed infection. It has been shown that a virus with a weak shutoff function competitively inhibits the strong shutoff effect of another strain (Hill et al., 1985) and a virus with stable IE mRNA increased the normally low stability of the IE RNA of a co-infecting virus (Fenwick & Clark, 1982, 1983). Therefore mixed infections were performed in order to investigate the dominance between viruses 17+ and 17G41.

BHK cells were infected with both 17+ and 17G41 and incubated with actinomycin before labelling. In this case 17+ prevented the shutoff of host protein synthesis by 17G41 (Fig. 4a, compare lanes 3 and 4). In another experiment (data not shown) 5 p.f.u. of 17+ per cell significantly reduced the shutoff effect of 17G41 infected at 10 p.f.u. Similarly, after mixed infection in the presence of CX, IE polypeptides of 17+ were made normally on removal of the inhibitor (Fig. 4a, lane 8). Thus the relative dominance of the UL41 alleles in the diploid situation is reversed in mixed infection. This observation is discussed below.

Inactivation of the original UL41 gene of 17G41

As the presence of the UL41 genes of both HSV-2(G) and HSV-1(17+) in virus 17G41 might cause some problems in the interpretation of its phenotype, the UL41 gene of 17+ in 17G41 was inactivated by insertion of a lacZ expression cassette into its coding region. The Hin-
Transfer of HSV UL41 gene

Fig. 5. Construction of pMF4. The 4.1 kb XbaI fragment containing the lacZ gene linked to the SV40 early promoter (lower line) was inserted into the UL41 gene of HSV-1(17+) (upper line, showing the region between 0.59 and 0.62 map units) at the NruI site. Numbers indicate restriction fragment sizes (kbp).

dIII/PstI fragment of HSV-1(17+) DNA that contains the gene UL41 was isolated from pGX80 and inserted into pUC9 to form plasmid pMF2. An XbaI linker was ligated into the unique NruI site in the UL41 coding region of pMF2 (see Fig. 5) to give pMF3. The lacZ expression cassette of pFJ3, which contains the lacZ gene linked to the SV40 early promoter, was isolated as a 4.1 kb XbaI fragment and inserted into the XbaI site of pMF3 to give pMF4 (Fig. 5).

Plasmid pMF4 was linearized by digestion with PstI and co-transfected into BHK cells with infectious 17G41 DNA. The resulting progeny were screened for viruses expressing lacZ by plaque assay under agar containing X-Gal. Blue plaques were picked and after further plaque purification viral genomes were examined to confirm the presence of the insertion in gene UL41. A stock of virus with the desired genotype was grown up and designated 17(41-)G41. An analysis of its DNA is shown in Fig. 6. The inserted 4.1 kb lacZ sequence, carrying two HindIII sites, divided the original 3.1 kb HindIII/PstI fragment of strains 17+ and 17G41 (Fig. 6, lanes 1 and 2) into two fragments of 1.8 and 1.6 kb and a lacZ fragment of 3.8 kb in 17(41-)G41 (lane 3). The bands at 2.4 and 1.2 kb in both 17G41 and 17(41-)G41 represent cross-hybridization between the UL41 sequences of HSV-1(17+) in the pMF4 probe and PstI fragments containing HSV-2(G) UL41 sequences at the TK locus (see Fig. 1). These bands are consistent with the retention of UL41 of strain G in 17(41-)G41, confirmed by the analysis of its TK region using a TK probe (Fig. 6, lanes 4 and 5).

Characteristics of 17(41-)G41

Growth of stocks of 17(41-)G41 from low multiplicity inocula consistently yielded titres approximately 10-fold lower than that of 17G41 or of 17+. Particle numbers were estimated by electron microscopy and the values obtained for the ratio of particles to p.f.u. were 64 for 17+, 70 for G, 52 for 17G41 and 330 for 17(41-)G41.

The phenotype of 17(41-)G41 was investigated by infecting BHK cells in parallel with viruses 17+, G and 17G41. The inhibition of cellular protein synthesis caused by exposure to 1000 particles per cell in the presence of actinomycin was the same with 17(41-)G41 infection as with G or 17G41 (data not shown) and the
pattern of protein synthesis 3 h after normal infection with 17(41−)G41 was indistinguishable from that induced by its parent 17G41 (Fig. 7, lanes 5 to 8). Similarly, after removal of CX the synthesis of IE proteins was much lower than in cells infected with 17+ (Fig. 7, lanes 1 to 4). These results confirm that the strong shutoff and weak IE protein production of G are due to the nature of its UL41 gene product.

Discussion

The mechanism of early suppression of cellular protein synthesis by a virion-associated factor is still not understood. Mapping of the vhs mutation in HSV-1(KOS) (Kwong et al., 1988) suggested that the shutoff function was encoded by gene UL41 (McGeoch et al., 1988). The aims of the work described in this paper were to determine whether the product of gene UL41 of HSV-2(G) encoded a strong shutoff function and whether the instability of viral IE RNA exhibited in the presence of CX was co-inherited with it. The observations that the recombinant viruses 17G41 and 17(41−)G41, [carrying the UL41 gene of HSV-2(G) in an HSV-1(17+) genome] strongly suppress host protein synthesis in the presence of actinomycin and make unstable IE mRNA in the presence of CX is a direct demonstration of the functions of the gene.

Virion-mediated shutoff by HSV involves the breakdown of polyribosomes, and is accompanied by the degradation of cellular mRNA. Possible roles for the UL41 protein are firstly that it is itself a nuclease, secondly that it causes the dissociation of ribosomes from mRNA, leaving it more vulnerable to attack by a pre-existing ribonuclease, or thirdly that it activates a cellular nuclease. The first of these is difficult to reconcile with the fact that weak shutoff is dominant in mixed infections (Hill et al., 1985) and the second is unlikely as the IE mRNA of many strains is stable in the presence of CX (i.e. before association with ribosomes), suggesting that viruses with a strong shutoff function must activate a cellular nuclease to account for the breakdown of mRNA. Whether such nuclease action is the direct cause of the inhibition of protein synthesis, although likely, is unproven (discussed by Strom & Frenkel, 1987). We sought evidence for such a mechanism but found that the breakdown of polysomes after infection in the presence of either actinomycin or CX yields ribosomes which are unstable at high ionic strength, unlike those released by treating polysomes with RNase in vitro (Fenwick & Walker, 1978 and unpublished results).

One of the reasons for initiating the present study was to test the hypothesis that some strains of HSV may encode a virion-associated function which specifically stabilizes viral IE mRNA. This suggestion was based on the observation that after mixed infection, HSV-1(F), which expresses stable IE mRNAs, stabilized the normally unstable IE mRNA of HSV-2(G) (Fenwick & Clark, 1982, 1983). It was observed later that the stability of IE mRNA was determined by a gene or genes between 0.58 and 0.65 map units and that if the dominance of mRNA stability over strong shutoff seen in mixed infection also determined the phenotypes of intertypic recombinant viruses, then the specific hypothetical mRNA-stabilizing factor mapped in this region (Fenwick & Owen, 1988). However the present results show that the opposite is true: in recombinants 17G41 and 17(41−)G41 the strong shutoff of HSV-2(G) is dominant. Therefore the earlier studies with intertypic recombinants merely confirm the location of the shutoff gene, UL41.

As mentioned earlier, a virus which lacked shutoff activity prevented the induction of shutoff by another strain (Hill et al., 1985; Fig. 4a, this paper). The protection of RNA seen in mixed infections was probably the result of competition between the products of different UL41 genes. It is known that the half-lives of all mRNAs of HSV-1(KOS) (1 to 2 h) were considerably increased by a mutation in the vhs gene, UL41 (Read & Frenkel, 1983; Kwong & Frenkel, 1987; Oroskar & Read, 1987, 1989) and the exceptional instability of the IE mRNA of HSV-2(G) in the presence of CX (half-life of about 15 min) may reflect the action of a strong shutoff protein on mRNA unprotected by ribosomes. The RNA accumulated in stable form after removal of CX or during normal infection (Fenwick & Owen, 1988). The apparent discrimination of the shutoff mechanism between host and viral protein synthesis may be explained by the hypothesis that the shutoff activity is short-lived (Fenwick & Owen, 1988) and degradation of mRNA induced by UL41 is more than counterbalanced by the increasing rate of transcription of viral mRNA while that of cellular mRNA is steady or declining.

An intriguing finding of this study was that with the diploid recombinant 17G41 (which contains both weak and strong alleles of UL41) the strong shutoff effect is dominant, whereas in mixed infections 17+ prevented 17G41-induced shutoff. How can the cis-dominance and trans-recessiveness of the strong UL41 gene product be explained? The trivial explanations, that the 17+ allele of UL41 is either not expressed in 17G41 or not incorporated into the virions, seem improbable but cannot be tested directly because the product of gene UL41 has not yet been identified either in the infected cell or in the virus particle. There is some evidence that the 17+ allele
Fig. 8. Hypothetical model of the action of a dimeric UL41 protein molecule. Each polypeptide of the homodimer of strong shutoff protein (A) has an attachment site (C) and a shutoff-initiating site (D). The homodimer of weak shutoff protein (B) has modified shutoff sites and the heterodimer (C) may be carried in the virions of diploid virus 17G41.

is expressed because its inactivation in 17(41-)G41 results in relatively poor growth.

If the product of UL41 causes the activation of an RNase, either directly through a protein–protein interaction or via an induction pathway, it is possible that in mixed infections the weak UL41 protein excludes an active shutoff protein from its site of action (Hill et al., 1985). However, the failure of the weaker UL41 protein to interfere with the effect of the stronger if both are incorporated into the same particle suggests that their spatial relationship affects their function. For example, at the molecular level, if the protein acts (and is present in the virion) as a dimer it is possible that the heterodimer is able to cause efficient shutoff although it could be excluded from its site of action by a homodimer (or even by a monomer) of weak shutoff protein, which might have an equal or enhanced affinity for the attachment site. Such a model is illustrated in Fig. 8. Alternatively, at the particle level, it is possible that shutoff is initiated while the particle remains intact, ensuring a particular arrangement of UL41 protein molecules, one of which forms an attachment to a cellular site while another triggers the activation of the RNase effect.

Further elucidation of the mechanism of host cell shutoff requires the characterization of the UL41 gene product, the study of its role in the virus particle and the identification of the features which distinguish the strong from the weak shutoff alleles. These experiments are in progress.

The authors are grateful to Professor J. H. Subak-Sharpe for encouraging and enabling M.L.F. to spend a short sabbatical in Glasgow and to Dr Frazer Rixon for critical reading of the manuscript. R.D.E. is a member of the Medical Research Council Virology Unit. M.L.F is supported by a research grant (ref. 14508/1.5) from the Wellcome Trust.

References


(Received 7 June 1989; Accepted 17 October 1989)