

Inactivation of the shutoff gene (UL41) of herpes simplex virus types 1 and 2

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Gene UL41 of herpes simplex virus type 1 (HSV-1) and the corresponding gene of HSV-2, which control the virion-mediated early suppression of cellular protein synthesis, have been inactivated by inserting a β -galactosidase expression cassette into their coding regions. The resulting recombinants grew well in tissue culture, although with the type 2 recombinant viral protein synthesis was slightly delayed. As a result of inactivation of UL41 host protein synthesis was not suppressed in the presence of actinomycin or early in

normal infection, although it declined at a late stage. Polyribosomes were not broken down early in infection, cellular DNA synthesis was not inhibited and in the presence of cycloheximide stable alpha (immediate early) mRNA accumulated, in marked contrast to that of the parent HSV-2 strain. Comparison of the proteins of purified virions of HSV-1 and shutoff-defective recombinant virus revealed discrepancies consistent with the presence of the UL41 gene product in the enveloped virion.

Introduction

The early suppression of cellular protein synthesis that follows infection with some strains of herpes simplex virus (HSV) is controlled by gene UL41 (Morse *et al.*, 1978; Kwong *et al.*, 1988; McGeoch *et al.*, 1988). The inhibitory agent, possibly the gene product itself, is carried in the virions and acts in the absence of virus gene expression (Nishioka & Silverstein, 1978; Fenwick & Walker, 1978).

In an earlier report (Fenwick & Everett, 1990) we described the transfer of a strong shutoff gene into the non-essential thymidine kinase (TK) gene of a strain with a weaker shutoff function and the subsequent inactivation of the original weak UL41 of the diploid recombinant by inserting a foreign gene (β -galactosidase) into it. This sequence of operations was chosen in case the UL41 protein was also an important structural component of the virion. Although experiments with mutants have shown that active shutoff is not an essential function for growth of the virus in tissue culture (Fenwick & Clark, 1982; Read & Frenkel, 1983), it is conceivable that mutant forms of UL41 protein might perform a structural role despite having lost their shutoff activity. However, we have found that the same technique of inactivation of UL41 by insertion can be applied to wild-type strains, yielding viable shutoff-defective viruses. Therefore the UL41 protein is not an

essential structural component. The construction and some characteristics of these viruses are described.

Methods

Viruses and cells. The wild-type HSVs used were HSV-1 strain 17 syn⁺ (Brown *et al.*, 1973) and HSV-2 strain G (Ejercito *et al.*, 1968). They are referred to in the text at 17 and G. Inactivation of gene UL41 produced the corresponding recombinant strains 17(41⁻) and G(41⁻). BHK C13 and Vero cells were grown in Dulbecco's MEM (Gibco) with 5% foetal calf serum. BHK cells were used unless otherwise stated.

Purification of virions. 2×10^7 cells were infected with 10 p.f.u./cell and incubated from 2 to 18 h post-infection (p.i.) in medium containing 1/10 of the normal concentration of amino acids, 0.5% serum and 1 μ Ci/ml of [¹⁴C]protein hydrolysate (Amersham). Cells were collected by centrifugation and broken by Dounce homogenization in 1.5 ml 0.01 M-phosphate pH 7.2. Nuclei were sedimented after adding sucrose to 6%, resuspended in 0.5 ml 0.4 M-NaCl/0.01 M-phosphate and disrupted by ultrasonication. The supernatant cytoplasmic fraction of the homogenate was combined with the culture medium and centrifuged for 1 h at 25000 r.p.m. The virus pellet was resuspended in 0.5 ml 0.4 M-NaCl/0.01 M-phosphate and both nuclear and cytoplasmic preparations were layered on 4.5 ml sucrose gradients (6 to 24% w/v in the same high salt buffer) and centrifuged for 15 min at 20000 r.p.m. before the collection of 0.5 ml fractions. Two central fractions that contained a peak of radioactivity were pooled, diluted with phosphate-buffered saline (PBS) and centrifuged for 90 min at 25000 r.p.m. The pellets were dissolved in 200 μ l of cell lysis buffer (2% SDS, 5% mercaptoethanol, 6% sucrose, 0.001% bromophenol blue, 0.05 M-Tris-HCl pH 7), heated for 2 min in boiling water and 60 μ l samples were fractionated by electrophoresis in SDS-polyacrylamide gels.

Plasmids. pFJ3 contains the *lacZ* (β -galactosidase) gene linked to the simian virus 40 (SV40) early promoter in a 4.1 kb sequence flanked by *Xba*I sites. It was kindly provided by Dr F. J. Rixon. pMF4 (Fenwick & Everett, 1990) consists of the *lacZ* expression cassette inserted at the unique *Nru*I site in the coding region of gene UL41 of HSV-1(17), cloned in pUC9. pGBglIIIn (Fenwick & Everett, 1990) is the *Bgl*II n fragment of HSV-2(G) DNA, which contains UL41, cloned in pUC9. pMF11 is the type 2 analogue of pMF4, containing the *lacZ* gene inserted into UL41 of HSV-2(G).

Recombinants. To produce recombinant progeny, cells were transfected with 1 μ g of linearized plasmid per 35mm Petri dish and superinfected 3 to 4 h later with 100 to 1000 p.f.u. of intact virus. Transfection, identification and isolation of β -galactosidase-positive recombinant viruses by growth in the presence of Xgal were described before (Fenwick & Everett, 1990).

Polysome analysis. Near-confluent monolayers of 2.5×10^6 BHK cells were chilled on ice and lysed in 0.8 ml of a solution of 1.5 mM-KCl, 2.5 mM-MgCl₂, 40 mM-phosphate buffer pH 7, 1% sodium deoxycholate, 1% Triton X-100 (Shell). Lysate (0.4 ml) was layered on a 4.8 ml gradient of 6 to 24% (w/v) sucrose and centrifuged in a swing-out rotor (Beckman SW55) before absorbance was monitored at 254 nm with a Gilford density gradient fractionator.

Other methods. Assay of shutoff of host protein synthesis and mRNA stability by gel electrophoresis and autoradiography of labelled proteins were also described before (Fenwick & Everett, 1990). DNA synthesis was measured by adding [*Me*-³H]thymidine (1 μ Ci/ml) to the growth medium. The trichloroacetic acid-insoluble radioactivity in cell lysates was collected on glass fibre filters for scintillation counting.

Nomenclature. In the two systems of naming of infected cell polypeptides commonly used, alpha polypeptides ICP 4, 0, 22 and 27 are immediate-early polypeptides Vmw 175, 110, 68 and 63, respectively. Their mRNAs are produced after infection in the presence of cycloheximide.

Results

Inactivation of the UL41 gene of 17

The procedure was similar to that used previously to inactivate the UL41 gene of 17 in a diploid virus (Fenwick & Everett, 1990). In plasmid pMF4 the *LacZ* gene has been inserted at codon 251 of the 489 codons of gene UL41 of 17. BHK cells were transfected with the linear form of pMF4, prepared by digestion with *Pst*I and superinfected 3 h later with 17 at a multiplicity of 10^{-3} p.f.u./cell. The resulting progeny were screened for the presence of the *lacZ* gene by infecting BHK cells with an agarose overlay containing Xgal. A blue plaque was picked and the virus subjected to a further six cycles of plaque purification in the presence of Xgal before growing a stock which was named 17(41⁻). The inserted 4.1 kb *lacZ* sequence has two *Hind*III sites, one near one end and one 0.3 kb from the other. The site of integration of the *lacZ* gene was confirmed by Southern blot analysis of a *Hind*III/*Pst*I digest of viral DNA, probing with nick-translated pMF4. The 3.1 kb *Hind*III/*Pst*I fragment of the parental virus, 17, which contains UL41, was

replaced by fragments of 1.8 and 1.6 kb in 17(41⁻) as described previously (Fenwick & Everett, 1990; Fig. 5 and 6).

Protein synthesis in cells infected by 17(41⁻)

The effects of infection with equal multiplicities of 17 and 17(41⁻) were compared. The autoradiographic patterns of protein synthesis 6 h after normal infection were similar (Fig. 1, lanes 3 and 4), but in the case of cells infected with 17(41⁻) (lane 4) a band above the ICP 22 marker was reduced and a band in lane 3 slightly below the ICP 27 marker was not detected in lane 4.

Efficiency of virion-associated host shutoff was examined by infecting in the presence of actinomycin. 17(41⁻) had no effect on cellular protein synthesis (Fig. 1, lanes 5 and 7), whereas 17 caused substantial inhibition within 2 h (lane 6). Despite this, the two viruses induced similar levels of immediate early protein synthesis after reversal of a cycloheximide block (lanes 9 and 10), although in the case of 17(41⁻) only a single band appeared in the position of ICP 22 (lane 10), suggesting a defect or slowness in the processing of this polypeptide (Fenwick *et al.*, 1980).

Virion proteins of 17(41⁻)

Since gene UL41 determines the shutoff power of the virus particle, it is likely that the gene product is carried in the virion (although its effect could be indirect, acting on some other virion component which in turn initiates shutoff). In order to compare the virion proteins of 17 and 17(41⁻), virus particles containing radioactive proteins were purified by sucrose gradient centrifugation. Electrophoresis of these proteins revealed no difference between the particles (nucleocapsids) extracted from nuclei, except for a relatively prominent polypeptide of low M_r in 17(41⁻) (Fig. 2, lanes 1 and 2), but the combined cytoplasmic and released (enveloped) virions showed differences similar to those described above in relation to total infected cell polypeptides. In the autoradiogram of 17(41⁻) proteins a band just below ICP 27 was missing and one above ICP 22 was less prominent (compare lanes 3 and 4). In addition, a pair of extra 17(41⁻) bands can be seen in lane 4, slightly ahead of the ICP 22 marker (lane 5).

Inactivation of gene UL41 of G

The pair of contiguous *Sst*I fragment of pGBglIIIn that contains gene UL41 of G (Fenwick & Everett, 1990) was cloned in pUC19 and the single *Pst*I site at codon 243 of UL41 (total length 492 codons) (Everett & Fenwick, 1990) was then replaced by an *Xba*I linker, producing

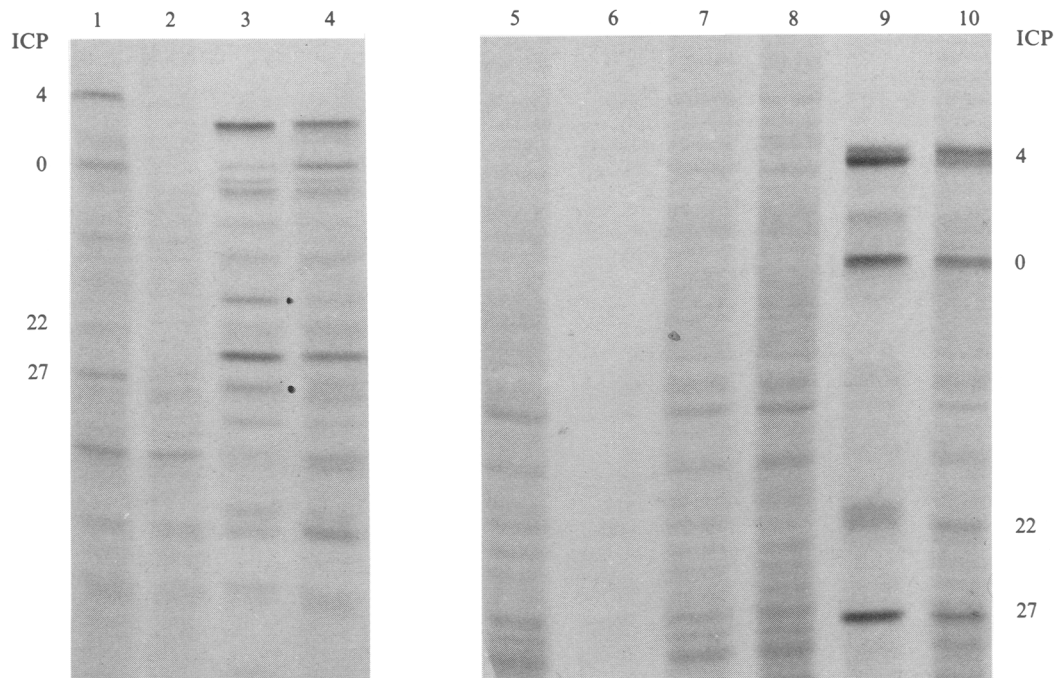


Fig. 1. Effect of 17(41⁻) on protein synthesis. Vero cells infected with 17 (lane 3) or 17(41⁻) (lane 4) were incubated with ¹⁴C-labelled amino acids from 6.5 to 7.5 h. Differences mentioned in the text are marked between lanes 3 and 4. Lane 1 shows α polypeptides ICP 4, 0, 22 and 27 as markers and lane 2 is a mock-infected control. Other cell cultures were labelled after infection for 2 h in the presence of actinomycin (lanes 5 to 7) or after 4.5 h in the presence of cycloheximide (lanes 8 to 10). Lanes 5 and 8, mock-infected; lanes 6 and 9, infected with 17; lanes 7 and 10, infected with 17 (41⁻). Autoradiograms of polyacrylamide gels are shown (lanes 5 to 10 are from a longer electrophoretic run than lanes 1 to 4).

pMF9. Linear forms of pMF9 obtained by partial digestion with *Xba*I (there is a second *Xba*I site in the pUC19 sequence of pMF9) were treated with alkaline phosphatase and ligated with the 4.1 kb *Xba*I fragment of pFJ3 which contains the *lacZ* gene linked to the SV40 early promoter. A plasmid with the desired insert in the UL41 gene was identified by digestion with *Sst*I and named pMF11.

BHK cells were transfected with linearized plasmid pMF11 and superinfected 4 h later with G, at 200 p.f.u. per 35 mm Petri dish. After a further 2 days incubation the mixed progeny were plated in the presence of Xgal. Blue plaques were picked and the galactosidase-positive virus was purified and grown up as before and designated G(41⁻).

The presence of the 4.1 kb *lacZ* sequence (with an *Sst*I site) in the expected position in UL41 was confirmed by Southern blot analysis. DNA extracted from cells infected with G(41⁻) was digested with *Sst*I and the blot was probed with nick-translated pMF9. The 2.2 kb fragment of G DNA that hybridized with the probe was replaced by two fragments of 2.9 and 3.4 kb in G(41⁻) DNA.

Protein synthesis and alpha mRNA stability in cells infected with G(41⁻)

The pattern of viral protein synthesis 3 h after infection with G(41⁻) was similar to that in cells infected with G (Fig. 3, lanes 2 and 3) but host synthesis was not shut off, as is seen clearly after infection in the presence of actinomycin (lanes 5 and 6). After reversal of a cycloheximide block, the production of ICP 4, 0 and 27 was greatly enhanced (compare lanes 8 and 9). ICP 22 is more difficult to detect by labelling (Fenwick *et al.*, 1980) but can be seen in Fig. 4, lanes 4 and 5. The stability of the alpha mRNAs in the presence of cycloheximide was assessed by the indirect method of measuring protein synthesis before and after a 1 h chase period with added actinomycin. Whereas the alpha mRNAs of G were no longer detectable after the chase (Fenwick & Clark, 1983; Fenwick & Owen, 1988), those of G(41⁻), like those of 17, were unchanged (Fig. 4).

Fig. 5 shows the autoradiographic patterns of proteins made at different times after infection with either G or G(41⁻). The progress of G(41⁻) infection appears to lag some 2 h behind that of G and, as noted above, G(41⁻)

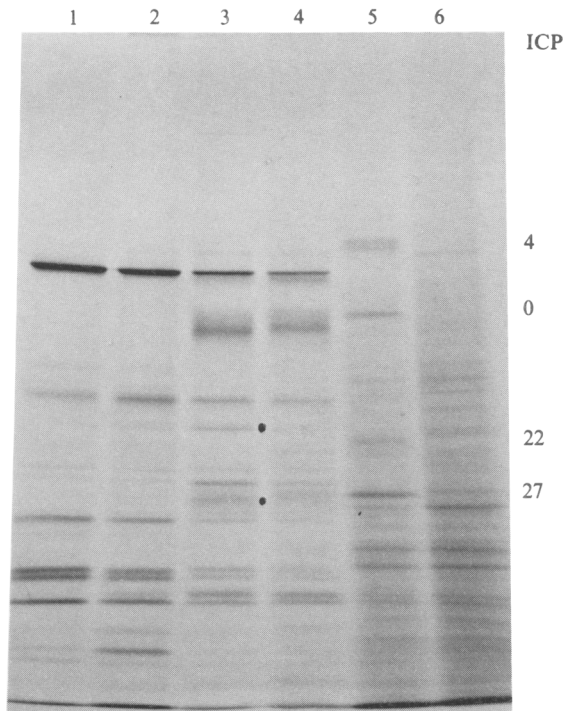


Fig. 2. Autoradiogram of virion polypeptides. Virions incubated with ¹⁴C-labelled amino acids were isolated from the nuclei (lanes 1 and 2) or from the combined cytoplasmic fraction and culture medium (lanes 3 and 4) of cells infected with 17 (lanes 1 and 3) or 17(41⁻) (lanes 2 and 4). After disruption with SDS they were subjected to electrophoresis. Lane 5 shows marker α polypeptides ICP 4, 0, 22 and 27 and lane 6 is the corresponding mock-infected control. Differences referred to in the text are marked between lanes 3 and 4.

has little effect on host protein synthesis in the early stages, although by 10 h a significant decline has occurred.

Breakdown of polyribosomes

Polyribosomes are disaggregated during the early shutoff of protein synthesis but it is not clear whether this precedes or follows the degradation of mRNA. It is conceivable that the disaggregation is a distinct effect of infection, unrelated to UL41, and that the released mRNA is then degraded. We therefore examined the state of aggregation of polyribosomes in lysates of infected cells by sedimentation in sucrose gradients. Infection with G for 90 min eliminated the polyribosomes and produced a large increase in single ribosomes and pairs, but the same multiplicity of G(41⁻) had very little effect (Fig. 6). Since a transient effect might have been missed, lysates were prepared 15, 30, 45 and 60 min after infection with G(41⁻) but no differences from mock-infected patterns were seen.

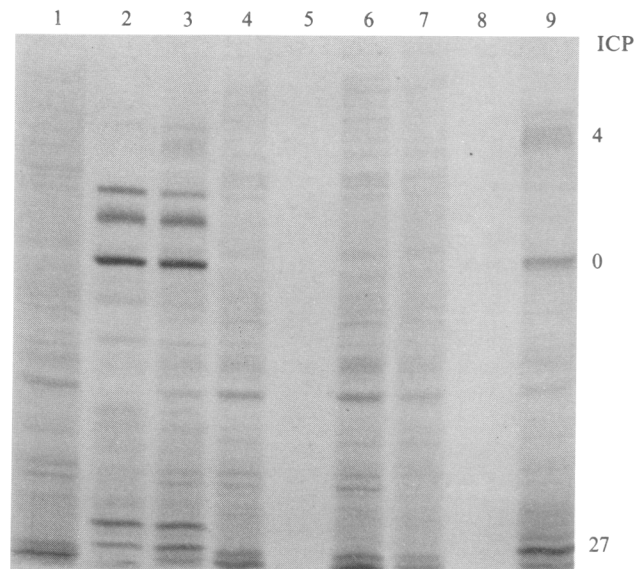


Fig. 3. Effect of G(41⁻) on protein synthesis. BHK cells were infected with G (lanes 2, 5 and 8) or G(41⁻) (lanes 3, 6 and 9) or mock-infected (lanes 1, 4 and 7) and incubated from 3 to 4 h with ¹⁴C-labelled amino acids. Lanes 4 to 6: actinomycin, 2 μ g/ml, was present throughout. Lanes 7 to 9: cycloheximide, 50 μ g/ml, was present from 0 to 3 h.

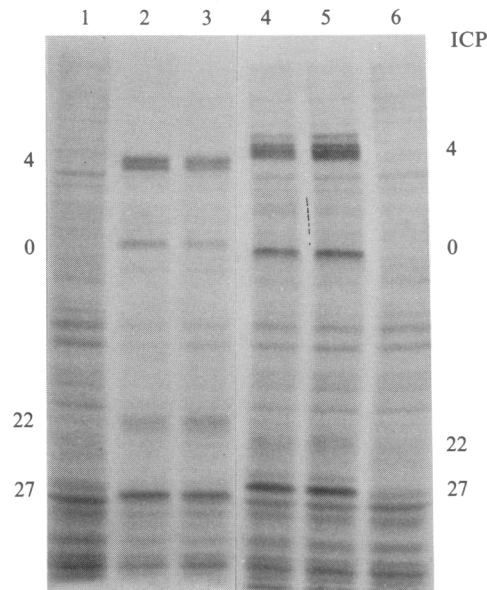


Fig. 4. Stability of the α mRNA of G(41⁻). Cells infected with 17 (lanes 2 and 3) or G(41⁻) (lanes 4 and 5) or mock-infected (lanes 1 and 6) were incubated with cycloheximide. At 3 h cycloheximide was removed and proteins were labelled for 1 h in the presence of actinomycin (lanes 1, 2, 4 and 6). To duplicate infected cultures (lanes 3 and 5) actinomycin was added at 3 h and incubation continued for a 1 h chase period before washing to remove cycloheximide and labelling as above. The autoradiogram was prepared after electrophoresis of labelled proteins.

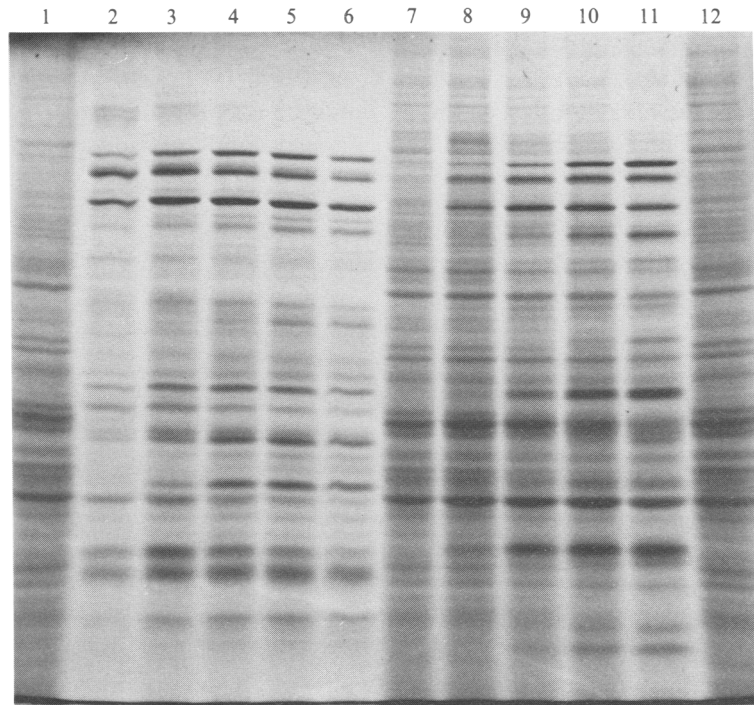


Fig. 5. Time course of infection with G or G(41⁻). Cells infected with G (lanes 2 to 6) or G(41⁻) (lanes 7 to 11) were incubated with ¹⁴C-labelled amino acids for 1 h in the presence of actinomycin, starting at: lanes 2 and 7, 1.5 h; lanes 3 and 8, 3 h; lanes 4 and 9, 5 h; lanes 5 and 10, 7 h; lanes 6 and 11, 10 h. Mock-infected cultures were labelled at 1.5 h (lane 1) and 10 h (lane 12).

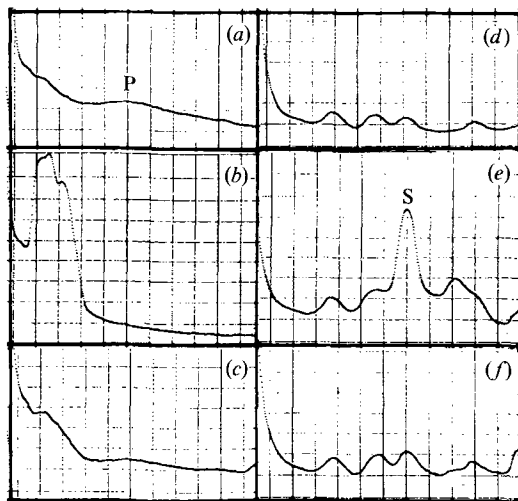


Fig. 6. Polysome breakdown. Cells were mock-infected (*a* and *d*) or infected with G (*b*, *e*) or G(41⁻) (*c*, *f*) and lysed 90 min later. The lysates were divided between pairs of sucrose gradients and centrifuged for 50 min at 30000 r.p.m. (*a*, *b*, *c*) or 65 min at 45000 r.p.m. (*d*, *e*, *f*) before scanning absorbance at 254 nm. Direction of sedimentation is from left to right. P, polysomes; S, single (74S) ribosomes.

DNA synthesis

The rate of cellular DNA synthesis was estimated by measuring the radioactive thymidine incorporated into acid-insoluble material between 1.5 and 2.0 h after

infection with 10 p.f.u./cell (i.e. well before the onset of viral DNA synthesis). The rate was reduced to 40% of the normal incorporation after infection with G but was unaffected by infection with G(41⁻).

Discussion

Since the shutoff gene can be interrupted by the insertion of a foreign sequence without seriously affecting the viability of the virus in tissue culture, its protein product is presumably not an essential structural component of the virus particle. However, a comparison of the proteins of purified virions of 17 and 17(41⁻) revealed differences that are consistent with the presence of the UL41 gene product in the enveloped virion. Two bands on the autoradiogram of labelled proteins prepared from mature particles of 17, representing proteins with apparent M_r values of approximately 75K and 58K, were missing from the pattern of 17(41⁻) proteins and similar discrepancies were seen among the total proteins from cells infected with 17 or 17(41⁻). The unprocessed produce of gene UL41 is expected to have an M_r of 55K, close to that of ICP 27 (McGeoch *et al.*, 1988). Its mRNA was detected and translated *in vitro* into a polypeptide of apparent M_r 58K (Frink *et al.*, 1981) and a polypeptide of similar size has been identified in infected cell lysates by

reaction with an antiserum raised against a synthetic peptide (J. R. Smiley & C. A. Smibert, personal communication).

During virion-mediated suppression of protein synthesis polyribosomes are dissociated and cellular mRNA is degraded (Fenwick & Walker, 1978; Fenwick & McMenamin, 1984; Mayman & Nishioka, 1985; Schek & Bachenheimer, 1985). The observation that G(41⁻) has no apparent effect on polysomes shows that the product of gene UL41 initiates polysome breakdown as well as mRNA degradation. It could do both by activating an RNase, as discussed by Strom & Frenkel (1987).

The ability to suppress cellular DNA synthesis was shown to be a function of the same region of the genome as shutoff of protein synthesis (Fenwick *et al.*, 1979). The failure of G(41⁻) to inhibit DNA synthesis confirms that both effects are functions of gene UL41. Cellular DNA synthesis, unlike viral, is dependent on concomitant protein synthesis and the likelihood that the decline of DNA synthesis after infection with G is a consequence of the inhibition of protein synthesis has been discussed (Fenwick, 1984).

In cells infected with G in the presence of cycloheximide, alpha mRNAs are very unstable, having half-lives of about 15 min (Fenwick & Clark, 1983; Fenwick & Owen, 1988) and consequently alpha proteins of G are relatively difficult to detect by labelling after removal of cycloheximide. With G(41⁻) the same mRNAs are stable and a much higher level of synthesis of alpha proteins is observed. This is the converse of the result we obtained earlier by inserting the UL41 gene of G into 17 (Fenwick & Everett, 1990), and together they provide a clear demonstration of the effect of a strong shutoff gene on viral mRNA stability described by others using the shutoff-defective mutant of HSV-1 (KOS), vhs-1 (Read & Frenkel, 1983; Oroskar & Read, 1987, 1989; Kwong & Frenkel, 1987; Kwong *et al.*, 1988).

However, in the course of normal infection with G the alpha mRNA, among others, is stable (Fenwick & Owen, 1988). This is probably not due simply to its association with ribosomes, because treatment of infected cells with levels of NaCl (0.15 M), NaF (0.015 M) or puromycin (50 µg/ml) that cause breakdown of polysomes in uninfected cells does not cause rapid degradation of viral mRNA (M. L. Fenwick, unpublished results). Similarly, in cells infected with G in the presence of cycloheximide, within 1 h of removing the inhibitor newly made alpha mRNA is stable (Fenwick & Clark, 1983); or if cycloheximide is added 1 h after infection stable alpha mRNA accumulates and is translated on removal of the drug at 3 h (M. L. Fenwick, unpublished results). These observations suggest that a virus-induced protein made early in infection is respon-

sible for protecting viral mRNA from the effects of the virion-borne UL41 gene product. The most conspicuous early proteins to be made soon after reversing cycloheximide are ICP5 (the major capsid protein), ICP6 (the large subunit of ribonucleotide reductase) and ICP8 (Fenwick & Clark, 1983). ICP8 has a strong affinity for single-stranded DNA and also for poly(rA) (Ruyechan & Weir, 1984). It is believed to have a number of different functions in the nucleus (Gao & Knipe, 1989) including that of down-regulating the expression of other viral genes (Godowski & Knipe, 1983) possibly by binding to mRNA. Godowski & Knipe (1983) did find, however, that after infection with a temperature-sensitive ICP8 mutant, ts13, at the permissive temperature the level of accumulation of ICP 4 mRNA was substantially less than with the wild-type virus, HSV-1(KOS), prompting the speculation that ICP8 might be the mRNA-stabilizing factor postulated above, counteracting the nuclease effect of the shutoff protein.

The benefit to the virus in conserving an indiscriminate shutoff mechanism involving the degradation of its own mRNA is not immediately obvious. It is possible that the gain in transcriptional control of the different classes of viral gene expression (Strom & Frenkel, 1987) might be a sufficient advantage. Other possibilities are that, once cellular mRNA has been reduced to a low level and its synthesis inhibited, the balance is tipped in favour of the virus by the relatively favoured transcription of viral DNA (C. A. Smibert & J. R. Smiley, personal communication) or by the production of an mRNA-stabilizing factor.

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