On the Control of Immediate Early (α) mRNA Survival in Cells Infected with Herpes Simplex Virus

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(Accepted 11 July 1988)

SUMMARY

The α or immediate early mRNA of herpes simplex virus strain HSV-2(G) had a half-life of about 15 min if made in the absence of viral protein synthesis but was relatively stable if viral protein synthesis occurred, either freely or restricted by the presence of the proline analogue azetidine. In contrast, the α mRNA of other strains of the virus is stable, even in the absence of protein synthesis. Studies with recombinant viruses showed that the region of the viral DNA between 0.58 and 0.65 map units [which includes the gene (vhs, UL41) that controls virion-mediated shutoff of host protein synthesis] is important in determining the survival of α mRNA. In mixed infection experiments HSV-2(G) inhibited α as well as host protein synthesis but the shutoff activity appeared to be short-lived. Within 3 h after infection, as a result of protein synthesis, cells became completely resistant to shutoff by superinfecting virus.

INTRODUCTION

The α or immediate early genes of herpes simplex virus (HSV) are transcribed by a cellular RNA polymerase in the absence of prior viral protein synthesis, i.e. if an inhibitor such as cycloheximide (CX) is added at the time of infection (Costanzo et al., 1977; Honess & Roizman, 1975). Measurements of rates of protein synthesis after blocking RNA synthesis with actinomycin (and removing the CX) led to the conclusion that the messenger function of the α mRNA of HSV-2(G) was short-lived (Fenwick & Clark, 1982, 1983), although that produced in the course of normal infection was relatively stable. This suggested that a message-preserving factor was made early in infection, possibly a viral β protein. On the other hand, the α mRNA of other strains, such as HSV-1(F) and HSV-2(HG52), was stable, even in the presence of CX. Possibly the hypothetical stabilizing factor was present in the virions, or the mRNA of these strains was intrinsically less susceptible to attack.

After first establishing that the α mRNA of HSV-2(G) is rapidly degraded unless viral protein synthesis occurs, we have tried to assess the influence of the structure of the RNA itself and of other viral genes in determining its stability.

Many strains of HSV suppress the protein synthesis of their host cells early in infection by a mechanism apparently mediated by a component of the virus particles (Nishioka & Silverstein, 1978; Fenwick & Walker, 1978) and accompanied by degradation of mRNA (Fenwick & McMenamin, 1984; Mayman & Nishioka, 1985; Schek & Bachenheimer, 1985). Studies with a mutant of HSV-1(KOS), vhs-1, which fails to shut off host protein synthesis, have shown that all of its mRNAs are appreciably more stable than those of the wild-type parent virus (Read & Frenkel, 1983; Oroskar & Read, 1987; Kwong & Frenkel, 1987; Kwong et al., 1988). HSV-2(G) has a strong virion-associated host shutoff activity, HSV-1(F) has less and HSV-2(HG52) has little or none. Therefore the role of the shutoff function in determining the survival time of the α mRNA of HSV-2(G) has been considered.
METHODS

Cells. Confluent 25 cm² monolayers of Vero (African green monkey kidney) cells were infected with an added multiplicity of 10 to 20 p.f.u./cell during 20 min at room temperature before removal of inoculum and incubation at 37 °C.

Viruses. HSV-1(F) and HSV-2(G) were obtained from B. Roizman, University of Chicago, Ill., U.S.A. and HSV-2(HG52) from J. H. Subak-Sharpe, University of Glasgow, U.K. They are referred to as F, G and HG52. Recombinants and their parental strains were kindly provided by B. Roizman (A8E, C2D; Morse et al., 1978) and A. J. Conley, Philadelphia, Pa., U.S.A. (R5a, RS1G25, RH1G7; Conley et al., 1981, Para et al., 1983).

RNA. Cytoplasmic poly(A)-containing RNA was isolated according to Maniatis et al. (1982), denatured with formaldehyde (6%, 15 min at 65 °C in 50% formamide), fractionated by electrophoresis in 1% agarose gels containing 6% formaldehyde and transferred to nitrocellulose by blotting in 10 × SSC.

DNA. HSV-2(G) DNA was extracted from infected Vero cells with SDS, treated with proteinase K and purified by centrifugation in sodium iodide gradients (Walboomers & ter Scheggett, 1976). The BamHI g fragment of HSV-2(HG52) DNA, cloned in pAT153, was provided by J. B. Clements, Glasgow, U.K.

Hybridization. 32P-labelled probes were made with the 'multiprime' system of oligonucleotide primers (Amersham). Nitrocellulose blots were treated with 5 × 10⁸ c.p.m./cm² in 50 µl 50% formamide for 16 h at 42 °C. After a final wash in 0.1 × SSC at 65 °C they were exposed to X-ray film with enhancer screens for up to 10 days at −70 °C.

Protein. Proteins were labelled by incubating cells with ¹⁴C-labelled amino acids (protein hydrolysate; Amersham) in amino acid-free medium for 1 h at 37 °C. After extraction with SDS they were separated by electrophoresis in 9% polyacrylamide-diallyltartardiamide–SDS gels for detection by autoradiography.

Inhibitors. Actinomycin (used at a concentration of 2 µg/ml), CX (50 µg/ml) and azetidine (1 mg/ml with one-tenth of the normal concentration of amino acids) were obtained from Sigma. CX was removed when necessary by washing cells twice with phosphate-buffered saline (PBS). Ultraviolet irradiation was done with a 15 W germicidal lamp 20 cm from 3 ml of virus diluted in PBS in a 9 cm diameter dish (10 s exposure, approximate dose 1 J/m²).

RESULTS

Stability of α mRNAs of wild-type viruses

The stability of virus-specific RNA was assessed by observing changes in levels during a 1 h chase period following the addition of actinomycin to stop RNA synthesis. The RNA from cells infected with G in the presence of CX produced three bands on the autoradiogram in the positions expected for mRNAs of ICP4, ICP0 and ICP22/27 (Watson et al., 1979) but after an actinomycin chase they were barely detectable (Fig. 1a, lanes 1 and 2). In contrast, the corresponding α mRNAs of F (lanes 3 and 4) were stable during the chase period.

Azetidine, a proline analogue, enhances the production of α proteins, probably by being incorporated into β proteins that are then unable to perform their normal function of switching off α protein synthesis (Honess & Roizman, 1975). Measurements of protein synthesis indicated that the α mRNA of G made in the presence of azetidine was stable (Fenwick & Clark, 1983). Apparently the supposed protein responsible for protecting the α mRNA from breakdown was still effective. The stability of the RNA was confirmed by hybridization as shown in Fig. 1(b).

The rates of degradation of α mRNA in the presence or absence of CX were compared by extracting RNA at shorter intervals during the chase period and hybridizing it with a probe specific for mRNAs for ICP4 and ICP0. These RNAs were stable in normal infection but had half-lives of the order of 15 min in CX-treated cells (Fig. 2).

These results show that the functionally unstable α mRNA of G made in the presence of CX is also physically unstable and reinforce our earlier conclusion that stable RNA is made only after viral β protein synthesis has started.

Stability of α mRNAs of recombinant viruses

If the stability of α mRNA were an inherent characteristic of the RNA itself it would be unchanged by transfer of an α gene from one strain of virus to another. R5a is a recombinant virus in which a small section of the genome of HSV-1(MP), carrying the gene for ICP27, has been replaced by the corresponding region of HSV-2(G) DNA. HSV-2 ICP27 can be recognized by its electrophoretic mobility being slightly slower than that of the type 1 protein. In the
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(a)

1 2 3 4

28S
ICP4
ICP0
18S
ICP22/27

(b)

5 6

Fig. 1. Stability of α mRNAs. Cells were infected with HSV-2(G) (lanes 1, 2, 5 and 6) or HSV-1(F) (lanes 3 and 4) in the presence of CX (lanes 1 to 4) or azetidine (lanes 5 and 6). Cytoplasmic poly(A)+ RNA was extracted at 3 h or after a further 1 h chase period in the presence of actinomycin (lanes 2, 4 and 6), blotted on to nitrocellulose after electrophoresis, and probed with 32P-labelled HSV-2 DNA. The positions of marker ribosomal RNAs (18S and 28S) are indicated. The autoradiogram shows viral mRNAs for α ICPs 4, 0 and 22/27.

(a)

1 2 3 4 1

2 3 4

(b)

Fig. 2. Stability of α mRNAs. Cells were infected with HSV-2(G) and incubated in the presence (b) or absence (a) of CX. Actinomycin was added at 3 h and cytoplasmic RNA was extracted 0, 15, 30 or 60 min later (lanes 1 to 4 respectively). The Northern blot was probed with 32P-labelled BamH1 g fragment of G DNA to identify mRNAs for α ICPs 4 and 0.

presence of CX the recombinant virus, unlike the type 2 parental strain, induced the production of stable ICP27 mRNA, which was translated on removal of the inhibitor (Fig. 3a, lanes 3 and 4). Therefore some part of the genome other than the α ICP27 gene itself controls mRNA accumulation and stability.

Other recombinant viruses have been examined in attempts to locate the controlling region. In recombinant RS1G25 a section of DNA of HSV-1(MP) between map units 0.58 and 0.70 is replaced by HSV-2(G) DNA. The α mRNAs of HSV-1(MP) accumulated and were stable in the presence of CX, but those of the recombinant were hardly detectable, although type 1 ICP4 was made normally in the absence of CX.
Recombinant virus A8E contains a section of HSV-1(17) DNA from approx. 0.43 to 0.65 map units inserted into the genome of HSV-2(G). This had the effect of eliminating the strong host shutoff activity (Morse et al., 1978) and the ICP4 and ICP0 mRNAs were stable (Fig. 3b). The electrophoretic mobilities of both ICP4 and ICP0 differed slightly from both parental forms, presumably due either to undetected changes in the genes concerned or in the post-translational modification of the polypeptides. The properties of RS1G25 and A8E together suggest that the stability of $\alpha$ mRNA in the presence of CX is determined by a gene or genes between approx. 0.58 and 0.65 map units.

Results obtained using two other recombinants were consistent with this conclusion. In RH1G7, insertion of HSV-2(G) DNA into an HSV-1(MP) genome between approx. 0.30 and 0.46 map units did not destabilize the type 1 $\alpha$ mRNA. Conversely, in recombinant C2D, insertion of type 1 DNA between 0.42 and 0.52 map units did not stabilize the type 2 $\alpha$ mRNA. A summary of the properties of the recombinants is given in Fig. 4.

**Non-specific shutoff**

The region between 0.58 and 0.65 map units contains, among others, the vhs gene, which determines the early shutoff power of the virus (Morse et al., 1978; Read & Frenkel, 1983;
Kwong et al., 1988). Since the process of shutoff is accompanied by degradation of cellular mRNA, it is relevant to ask whether the shutoff mechanism of G is specific for cellular protein synthesis or whether it can also act against viral (i.e. its own) protein synthesis, possibly destroying viral mRNA, but with diminishing effect as the mRNA-stabilizing factor is produced.

After simultaneous infection with F and G the strong vhs activity of G did not reduce the synthesis of F proteins, suggesting that the inhibition applied specifically to host protein synthesis, although G was able to shut off the synthesis of both viral and cellular proteins in cells infected 7 h previously with the paramyxovirus Sendai virus (Fenwick & Walker, 1978). Similarly, in cells infected in the presence of CX with the shutoff-defective HSV-1 mutant vhs-1, and later washed and superinfected with wild-type virus in the presence of actinomycin, both cellular and mutant viral protein syntheses were suppressed (Read & Frenkel, 1983). A similar experiment was therefore done with strains G and HG52. In cells infected with HG52 in the presence of CX, superinfection with G plus actinomycin immediately after removing CX prevented both α-HG52 and cellular protein synthesis (Fig. 5a, lanes 3, 4 and 7, 8), confirming that shutoff can be non-specific.

In contrast, if CX was omitted during the first infection, superinfecting G had only a partial shutoff effect at 2 h (Fig. 5a, lanes 5 and 6) and no effect on either cell or virus at 3 h (lanes 1 and 2). Evidently the cells acquired a resistance to shutoff as a result of protein synthesis. The protein concerned is probably a viral β or γ protein, because the cells remained susceptible to general shutoff if α protein synthesis only was allowed, i.e. by incubating in the presence of actinomycin after reversing a CX block (Fig. 5b).

**Transient shutoff**

If virion-mediated shutoff is non-specific, the failure of G to suppress the synthesis of other viral proteins after simultaneous mixed infection (Fenwick & Walker, 1978) suggests that the
early shutoff activity is transient. This is illustrated in Fig. 6, which shows the effect of G that had been inactivated by irradiation with u.v. light in order to avoid confusion between the synthesis of G proteins and those of co-infecting HG52.

Inactivated G shuts off host protein synthesis without making any proteins of its own, whereas HG52 makes viral proteins without switching off the host (Fig. 6, lanes 1 to 3). Simultaneous infection with both viruses resulted in partial host shutoff, but HG52 protein synthesis was slightly enhanced (Fig. 6, lane 4). Reduced shutoff is usually observed after such mixed infections, as was reported by Hill et al. (1985). Inactivated G added up to 3 h before HG52 suppressed host protein synthesis more strongly but still failed to inhibit HG52 protein synthesis. Similarly, if CX was present after simultaneous infection, when the inhibitor was removed HG52 α protein synthesis started (Fig. 6, lanes 5 and 6), indicating that the shutoff function of G was no longer operative.

The decline of shutoff activity was necessarily measured in mixedly infected cells. If the same decline occurs in singly infected cells and the shutoff of cellular protein synthesis and the degradation of α mRNA were effects of a common cause, the RNA would be expected to become progressively more stable as the shutoff activity declined. However, as shown above, after 3 h in the presence of CX the α mRNA of G is still very unstable and we saw no evidence of increased stability when the time of incubation was extended from 3 to 6 h.
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Fig. 6. Transient shutoff effect of u.v.-inactivated HSV-2(G). Cells were mock-infected (lane 1) or infected with u.v.-inactivated G (lane 2), HG52 (lane 3) or inactivated G plus HG52 (lane 4). Lane 5 represents cells infected with HG52 in the presence of CX and lane 6 cells infected with G plus HG52 in the presence of CX. At 3 h they were washed and incubated for 1 h with 14C-labelled amino acids and proteins were prepared for autoradiography.

DISCUSSION

Exactly how the HSV virion-mediated suppression of host protein synthesis is accomplished is not yet clear. The early breakdown of polysomes is accompanied by degradation of cellular mRNA although there is some doubt as to whether this is the cause of polysome breakdown. The resulting single ribosomes dissociated into subunits at high ionic strength, unlike those released by RNase (Fenwick & Walker, 1978) and there are reports of inhibition of protein synthesis preceding the loss of mRNA (Nishioka & Silverstein, 1978; Mayman & Nishioka, 1985; Schek & Bachenheimer, 1985).

The inhibition is selective in the sense that it affects the protein synthesis of the host but allows that of the virus, or of a co-infecting related virus, to proceed. This specificity appears not to be determined by the structure of the mRNA, since a haemoglobin gene incorporated into an HSV genome was actively expressed when synthesis of the analogous host protein was shut off (Smiley et al., 1987). It may be a temporal specificity, determined by the transience of the shutoff effect and the timing of viral mRNA synthesis, because in cells infected with HSV-2(HG52) there is a critical period during which the shutoff function of HSV-2(G) can be shown to act nonspecifically. It does not discriminate against cellular protein synthesis when infecting cells that are making both viral α and cellular proteins (Fig. 5). If the cells are already making later viral proteins as well as cellular proteins, G is unable to shut off either.
The product of the vhs gene of HSV-1(KOS) (probably gene UL41 of McGeoch et al., 1988) determines early shutoff and also reduces the stability of all viral mRNAs (Morse et al., 1978; Read & Frenkel, 1983; Oroskar & Read, 1987; Kwong et al., 1988). Our experiments with recombinant viruses have shown that the stability of the α mRNA of G in the presence of CX is controlled by the region of the genome between about 0.58 and 0.65 map units, within which the vhs gene is located (Kwong et al., 1988). However, although mixed infection with HG52 and u.v.-inactivated G indicated that the shutoff activity of G declines substantially within 3 h, we saw no evidence of a corresponding increase in stability of α mRNA.

Other observations have implied a more positive control of the stability of the α mRNA of G by a later viral protein (Fenwick & Clark, 1982, 1983). Stabilization of mRNA may also be involved in determining the extent of shutoff of host protein synthesis. For instance, protection of cellular mRNA by a virion protein might explain the observation that after mixed infection with a strongly and a weakly shutting off virus, the weaker was dominant (Hill et al., 1985), competitively inhibiting shutoff by the other virus. Similarly, production of a stabilizing factor might account for the development of resistance to shutoff as viral protein synthesis proceeds (Fig. 5).

If, as mixed infection experiments have suggested, the mRNA-stabilizing effect is dominant over the shutoff effect, the results with recombinant viruses reported here imply that the stabilizer is the product of a gene between 0.58 and 0.65 map units. This region of HSV-1 DNA contains, in order, genes for the large and small subunits of ribonucleotide reductase (RR1 and RR2), the host shutoff determinant (vhs, UL41), a 65K DNA-binding protein (Marsden et al., 1987), an unknown protein and the envelope glycoprotein gC. RR1 (also known as ICP6 or Vmw136) can probably be excluded as the stabilizing factor because of two of our unpublished observations. First, a comparison of electrophoretic mobilities suggests that recombinant RS1G25 (see Fig. 4) makes a type 1 RR1, whereas its α mRNA stability is characteristic of the HSV-2 parent (G). Secondly, a temperature-sensitive mutant, ts1207 (Preston et al., 1984), with a mutation in the RR1 gene, makes stable α mRNA in the presence of CX at the non-permissive temperature. Others are unlikely candidates because they have probable functions already assigned (RR1, RR2) or are expressed late (gC).

As a working hypothesis, we suggest that infection causes indiscriminate dissociation of ribosomes from mRNA which is then susceptible to degradation, possibly by a cellular nuclease, unless protected by the product of a gene in the 0.58 to 0.65 map unit region of the viral DNA. Further evidence for or against the operation of positive control of mRNA stability is being sought.

The correlation between the vhs-1 mutation, decreased host shutoff and increased stability of viral mRNA might be reconciled with the apparently dominant characters of weak shutoff (Hill et al., 1985) and α mRNA stability if the UL41 gene product itself were a protector of mRNA rather than a shutoff factor (with enhanced activity in the vhs-1 mutant), positive shutoff being initiated by another virion component or even by the virions themselves.

We are grateful to A. J. Conley, V. G. Preston, B. Roizman and J. B. Clements for viruses and cloned DNA, and to the Wellcome Trust for financial assistance (research grant 14508/1.5).

REFERENCES


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*(Received 31 March 1988)*