The Effect of Cycloheximide on the Accumulation and Stability of Functional α-mRNA in Cells Infected with Herpes Simplex Virus

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SUMMARY

Cells were infected with herpes simplex virus type 2, HSV-2(G), and incubated in the presence of cycloheximide (CX). When CX was removed and actinomycin D (Act D) added, α-polypeptides ICP 0 and ICP 4 were synthesized at low rates. If CX was removed without adding Act D, the rate of production of ICP 4 increased while that of ICP 0 remained constant. In cells treated with azetidine to enhance the production of ICP 4 and 0, accumulation of functional mRNA for ICP 4 (determined indirectly by translation in vivo) was reduced by concentrations of CX between 0.5 and 5.0 μg/ml, whereas mRNA for ICP 0 was unaffected by 50 μg/ml CX. CX apparently either inhibits the synthesis of ICP 4 mRNA or enhances its inactivation without affecting the production or degradation of ICP 0 mRNA. The accumulation of ICP 4 or ICP 0 mRNA of HSV-1(F) was unaffected by CX. The low levels of ICP 4 and ICP 0 mRNAs of HSV-2(G) that accumulated in the presence of CX disappeared rapidly after adding Act D, in contrast to those of HSV-1(F) which were stable. The ICP 4 mRNA of HSV-2(G) was stable, however, if made without CX or if in mixed infection with HSV-1(F) in the presence of CX. It is suggested that rapid inactivation may account for the low level of accumulation of functional ICP 4 and ICP 0 mRNAs of HSV-2(G) in the presence of CX, and that ICP 4 mRNA is protected by a protein made soon after normal infection. Such a protein may be carried in the virion of HSV-1(F).

INTRODUCTION

The 'immediate-early' or α-genes of herpes simplex virus type 1 (HSV-1) are expressed soon after entering a host cell and their protein products are needed to initiate the expression of other (β and γ) genes (Honess & Roizman, 1974, 1975). If protein synthesis is prevented by adding cycloheximide (CX) at the time of infection, α-mRNA is made, probably with the help of a cellular polymerase (Costanzo et al., 1977). It is then translated into at least four α-polypeptides, ICP (infected cell polypeptide) 4, 0, 22 and 27, if the inhibition is reversed (Honess & Roizman, 1974; Fenwick et al., 1980; Mackem & Roizman, 1981). The synthesis and translation of β- and γ-mRNA soon follows unless further RNA synthesis is prevented by adding actinomycin. A similar, although less stringent, restriction is imposed on transcription if the amino acid analogues canavanine or azetidine are incorporated into the α-proteins, rendering them inefficient in switching on β-mRNA synthesis (Honess & Roizman, 1975). In fact, in these artificial situations the rate of synthesis of α-proteins is greater than in untreated infected cells because one of the functions of normal β-proteins is to suppress the synthesis of α-proteins.

Using a type 2 virus, HSV-2(186), Powell & Courtney (1975) found that polypeptides similar to ICP 4 and ICP 0 were made soon after reversing CX inhibition in the absence of actinomycin, but that their rates of synthesis were not higher than in untreated infected cells and did not increase with longer CX treatment. We have observed that in cells infected with HSV-2(G) in the presence of azetidine (to enhance α-polypeptide production) CX prevents the accumulation of mRNA for ICP 4 and ICP 27, but not that of ICP 0 mRNA (Fenwick & Clark, 1982). It was
suggested that the mRNAs for ICP 4 and ICP 27 were either not made in the presence of CX or, if made, were functionally unstable. This paper presents further information concerning the effect of CX on the accumulation and stability of α-mRNA of HSV-2(G).

METHODS

Cells. Confluent monolayers of African green monkey kidney (Vero) cells were grown in 25 cm² tissue culture flasks and infected during 20 min at 20 °C with 10 to 20 p.f.u./cell in 1.5 ml growth medium. The inoculum was replaced by fresh growth medium and incubation at 37 °C started at zero time.

Viruses. Samples of HSV-1(F), HSV-1(HFEM), HSV-1(MP), HSV-2(G) and HSV-2(186) were obtained from Dr B. Roizman, University of Chicago, and HSV-2(HG52) from Professor J. H. Subak-Sharpe, University of Glasgow.

Azetidine (Calbiochem) was dissolved in growth medium containing 1/10 the normal concentration of amino acids. CX was also obtained from Calbiochem and actinomycin D (Act D) from BDH.

Labelling with ¹⁴C-amino acids (Amersham International) in the presence of Act D (2 µg/ml), and electrophoresis of proteins in SDS-polyacrylamide gradient gels were described previously (Fenwick et al., 1978).

RESULTS

Accumulation of α-mRNA during and after CX treatment

Monolayers of cells were infected with either HSV-1(F) or HSV-2(G) (hereafter referred to as F and G respectively) and incubated with or without CX for 4 h. They were then washed and labelled with ¹⁴C-amino acids in the presence of Act D to detect functional mRNA that had

![Fig. 1. Polypeptides synthesized after removal of CX in cells infected with F(a, b) or G(c, d). CX (50 µg/ml) was added to one culture of each pair (b, d) immediately after infection. All cultures were washed at 4 h post-infection and incubated for 1 h with ¹⁴C-labelled amino acids in the presence of Act D (2 µg/ml). (The same labelling procedure was used in all subsequent figures.) Cell lysates were subjected to electrophoresis and an autoradiogram prepared from the dried gel. ICP, Infected cell polypeptide.](image-url)
Fig. 2. Accumulation of mRNAs of G during and after incubation with CX. CX was removed 4 h after infection with G, and cells were either labelled with 14C-amino acids (a) or incubated for a further 1 h before labelling at 5 h post-infection (b). After electrophoresis the autoradiogram was scanned with a recording densitometer.

accumulated. Autoradiograms of the electrophoretically separated polypeptides showed that after reversal of the CX inhibition ICP 4, 0, 22 and 27 of F were made at higher rates than in normal infection (Fig. 1a, b), while in cells infected with G the rate of synthesis of ICP 4 was lower than normal (Fig. 1c, d). ICP 0 is not clearly distinguishable from ICP 8 in Fig. 1(c). Both ICP 4 and ICP 0 were relatively lightly labelled compared to the corresponding polypeptides of F, and neither ICP 22 nor ICP 27 were detected. After increasing periods of incubation with CX (between 2 and 4 h), the rates of production of ICPs of F increased progressively, but those of G did not change, as reported by Powell & Courtney (1975).

If Act D was not added when CX was removed from cells infected with G, the rate of synthesis of ICP 4 (as well as a number of β- and γ-polypeptides) increased but that of ICP 0 remained constant (Fig. 2).

These results suggest that steady low levels of functional mRNA for ICP 4 and ICP 0 of G are reached in the presence of CX, and that when CX is removed the level of ICP 4 mRNA begins to rise while that of ICP 0 mRNA does not.

Effect of CX in the presence of azetidine

The above suggestion is consistent with the results of experiments using azetidine to enhance α-polypeptide synthesis (Fenwick & Clark, 1982) which showed that CX restricts the accumulation of mRNA for ICP 4 and ICP 27 of G but not that for ICP 0. The experiment illustrated in Fig. 3 extends this observation, showing the effects of different concentrations of CX in the presence of azetidine. The appearance of mRNAs for γ-ICP 5 and β-ICP 6 and ICP 8 was substantially reduced by as little as 0.5 μg/ml CX. Accumulation of ICP 4 mRNA was slightly less sensitive, but that of ICP 0 mRNA was hardly affected by up to 50 μg/ml CX. The ratio of the areas of the peaks of ICP 4 and ICP 0 fell from 1.70 without CX to 0.53 with 50 μg/ml CX. The effect of CX on the translation of ICP 0 mRNA was evidently fully reversible, and presumably the low level of synthesis of ICP 4 after removal of CX was due to lack of functional
mRNA. It is noticeable, however, that whereas the peaks of ICPs 5, 6 and 8 disappeared altogether with increasing concentration of CX, that of ICP 4 showed little further decline between 5.0 and 50 μg/ml CX. A low level of ICP 4 mRNA was apparently maintained in the presence of CX. This may indicate that accumulation is not absolutely dependent on on-going protein synthesis, or it may simply be a reflection of the incomplete (approx. 95%) inhibition of protein synthesis by CX (Mackem & Roizman, 1981).

**Stability of mRNA**

In cells infected with G in the presence of azetidine it is probable that neither the synthesis nor the stability of ICP 0 mRNA is affected by CX, since it was translated normally when CX was removed, but ICP 4 mRNA is either not made in the presence of CX or, if made, is rapidly inactivated.

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**Fig. 3.** Effect of different concentrations of CX on accumulation of mRNAs of G. Cells infected with G were incubated with 6 mM-azetidine and 1/10 the normal concentration of amino acids in the presence of CX as follows: (a) none; (b) 0.5 μg/ml; (c) 5.0 μg/ml; (d) 50 μg/ml. The cells were washed and labelled at 3 h post-infection. Densitometer scans of the autoradiograms are shown.
It was stated earlier (Fenwick & Clark, 1982) that the ICP 4 mRNA of G that accumulates in the presence of azetidine is not less stable than that of F. In order to determine whether CX affects the stability of ICP 4 mRNA of G, Act D was added at 3 h, 1 h before removing CX and adding \(^{14}\text{C}\)-labelled amino acids. Neither ICP 0 nor ICP 4 became detectably labelled (Fig. 4, compare c and d). In contrast, the ICP 4 mRNA of G made in untreated cells (Fig. 4a, b) or in azetidine-treated cells (Fig. 4e, f) deteriorated only slightly during a 1 h chase period in the presence of Act D. The \(x\)-mRNAs of F that accumulate in the presence of CX are also relatively stable compared to those of G as shown in Fig. 5. Thus, CX appears to render ICP 4 mRNA of G unstable, but not that of F. ICP 0 mRNA of G (but not F) is also unstable if made in the presence of CX but, as mentioned at the beginning of this section, other experiments indicated that its stability is probably not affected by CX.

The ICP 4 mRNA of G that begins to accumulate after reversing CX inhibition (see Fig. 2) was functionally stable as shown in Fig. 6(a, b). Since host cell protein synthesis has been substantially inhibited by the virus by this time, this suggests that a newly made virus protein may be responsible for enhancing the stability of ICP 4 mRNA after reversal of CX.

The type 1 and type 2 forms of ICP 4 are distinguishable, having different electrophoretic mobilities. In cells infected with both F and G and incubated with CX, the synthesis of ICP 4 of G (after reversal of the CX block) was enhanced (Fenwick & Clark, 1982), suggesting that F contributed a factor that increased either the production or the stability of ICP 4 mRNA of G. Therefore, we examined its stability in mixedly infected cells, adding Act D 1 h before removing CX and labelling. The autoradiogram in Fig. 6(c, d) shows that the ICP 4 mRNA of G did not decline during the chase period, in contrast to that made in singly infected cells (Fig. 5). Apparently, F had a stabilizing influence on the mRNA of G in the presence of CX.
Fig. 5. Stability of α-mRNAs of F and G. Cells infected with either F(a, b) or G(c, d) were incubated with CX. One flask of each pair was washed and labelled at 3 h (a, c) and the other flask of each pair was treated with Act D at 3 h and then washed and labelled at 4 h (b, d). Films were exposed for 1 day (a, b) or 7 days (c, d).

Other viruses and cells

A number of other strains of HSV were examined to see whether they resembled F or G in their response to CX treatment, i.e. whether the accumulation of ICP 4 mRNA was enhanced or inhibited by CX. Two type 1 strains, HFEM and MP, resembled HSV-1(F) in that production of ICP 4 after reversal of a CX block was enhanced as compared to that in normally infected cells, whereas with two type 2 strains, 186 and HG52, it was reduced. In cells exposed to azetidine, CX had effects on ICP 4 production varying from little or none with F and HFEM, through partial inhibition with HG52, to almost complete inhibition with G and 186 (Fig. 7).

Two other types of host cell, BHK and HEp2, were also infected with F or G with results similar to those obtained with Vero cells, i.e. CX had little effect on the accumulation of ICP 4 mRNA of F in the presence of azetidine but substantially inhibited that of G.

Experiments in which cells were co-infected with F and various type 2 viruses showed that F also enhanced the production of ICP 4 of 186 (Fig. 8g) as well as that of G (Fig. 8c), but not that of HG52 (Fig. 8e). The degree of enhancement was variable and was not increased by increasing the multiplicity of infection of F. No enhancement of ICP 4 production was seen after simultaneous infection with both type 2 viruses, G and 186 (not shown). There was also some enhancement by F of the synthesis of ICP 0 of HSV-1(186), which is more clearly seen on the autoradiogram than in Fig. 8(g). The synthesis of ICPs 4, 0, 22 and 27 of F was reduced in mixed infection with 186 but not with G.
Fig. 6. Stability of mRNAs of G made after reversal of CX (a, b) or in mixed infection in the presence of CX (c, d). (a, b) CX was removed 2 h after infection with G and incubation continued. One flask was labelled at 4 h post-infection (a), and Act D was added to the other flask at 4 h post-infection, followed by labelling at 5 h post-infection (b). (c) Cells were infected with both F and G simultaneously (10 p.f.u./cell of each) and treated with CX. One flask was washed and labelled at 3 h (c), and the other flask was treated with Act D at 3 h post-infection and labelled at 4 h post-infection (d).

Fig. 7. Effect of CX on accumulation of α-mRNAs in the presence of azetidine. Pairs of flasks were infected with HSV-1 strains F, HFEM or MP, or HSV-2 strains HG52, 186 or G and incubated in medium containing 6 mM-azetidine and 1/10 the usual concentration of amino acids, with (b, d, f, h, j, l) or without (a, c, e, g, i, k) CX. At 5 h all were washed and labelled. The figure is composed of autoradiograms prepared from several gels. The ICP 0 bands are arbitrarily aligned. Since HG52 does not suppress cellular protein synthesis, the cells infected with HG52 were simultaneously exposed to u.v.-inactivated HSV-2(G) in order to reveal more clearly the α-polypeptides of HG52 (compare Fig. 8d).
DISCUSSION

The accumulation of functional mRNA for ICP 4 of HSV-2(G), in contrast to that of HSV-1(F), is reduced by CX. After removing CX, protein synthesis is again possible at normal rates and stable ICP 4 mRNA starts to accumulate (unless Act D is added), while the level of ICP 0 mRNA remains steady. However, cell protein synthesis is specifically suppressed, by an unknown mechanism apparently mediated by a component of the infecting virions (Fenwick & Walker, 1978), and this effect is not reversed when the cells are washed to remove CX. This suggests that the protein that promotes the accumulation of ICP 4 mRNA is not a host protein but an early virus protein. The most obvious candidates, in order of appearance after removing CX, are ICP 0, ICP 6 and ICP 8. That a virus-specific protein may be involved is supported by the observation that co-infection with F enhanced the accumulation of ICP 4 mRNA of G in the presence of CX [and probably also the accumulation of ICP 0 mRNA of HSV-2(186), as seen in Fig. 8]. It should be mentioned that the identification of this accumulating ICP 4 mRNA depends on the increased labelling of a polypeptide migrating during electrophoresis like ICP 4 of G.

Two type 1 viruses, strains HFEM and MP, behaved similarly to HSV-1(F) in that mRNA for ICP 4 (as well as for ICPs 0, 22 and 27) accumulated in the presence of CX, while HSV-2(186) resembled HSV-2(G). However, HSV-2(HG52) exhibited intermediate properties, and in view of the basic similarity between the structures of the DNAs of type 1 and type 2 viruses, and their patterns of transcription and protein synthesis during growth (Morse et al., 1978; Preston et al., 1978; Easton & Clements, 1980) it seems very unlikely that they differ fundamentally in their regulation mechanisms.

Either the production or the stability of functional ICP 4 mRNA of G is reduced by CX, and we have suggested previously (Fenwick & Clark, 1982) that a 'pre-α' protein might be involved in stimulating the production of ICP 4 and ICP 27 mRNAs. However, in the absence of measurements of transcription rates, our observation that the small amount of mRNA that does
accumulate in the presence of CX is less stable than that made without CX suggests that the RNA may be transcribed normally with CX but rapidly degraded. ICP 0 mRNA made in the presence of CX was also unstable but, unlike ICP 4 mRNA, it did not increase when CX was removed and neither was its accumulation in the presence of azetidine reduced by CX. Therefore, we suggest that in cells infected with G a protein, probably a virus-specific early protein, is made that prevents the inactivation of functioning (i.e. cytoplasmic) ICP 4 mRNA but not that of ICP 0 mRNA, and that in cells mixedly infected with G and F in the presence of CX both ICP 4 and ICP 0 mRNAs of G are protected to some extent by proteins carried in the virions of F.

In interpreting our observations in this way we have made certain assumptions. In using an indirect measure of the level of functional mRNA we are assuming that mRNA limits the rate of protein synthesis in vivo. That this is reasonable was borne out in previous experiments on the effect of CX in azetidine-treated cells (Fenwick & Clark, 1982) in which the results of in vitro translation were similar to those obtained by labelling proteins in intact cells. Our assumption that the inhibitory effect of CX on protein synthesis is as reversible in cells infected with F or G as in uninfected cells is supported by the observation (Fenwick & Clark, 1982, and Fig. 3 and Fig. 7, this paper) that in azetidine-treated infected cells the incorporation of 14C into α-ICPs of F and ICP 0 of G were the same whether or not CX was added at the start of infection and removed before labelling.

In uninfected cells the synthesis of certain mRNAs can be stimulated by exposing the cells to abnormally high temperatures. CX does not prevent the accumulation of this mRNA in chick fibroblasts (Kelley & Schlesinger, 1978) and we have observed (M. L. Fenwick & J. Clark, unpublished results) that the new mRNA induced in Vero cells by heat shock in the presence of CX is not particularly unstable. It is possible that the cells have a means of specifically inactivating foreign mRNA (unless it is protected) without affecting endogenous mRNA.

REFERENCES


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