Synthesis of \( \alpha \) (Immediate-Early) Proteins in Vero Cells Infected with Pseudorabies Virus

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SUMMARY

The synthesis of \( \alpha \) (immediate-early) polypeptides in Vero cells infected with pseudorabies virus was studied. Cycloheximide was added at the beginning of infection and removed several hours later. The accumulated \( \alpha \) mRNA was translated either \textit{in vivo} in the presence of actinomycin D to prevent further mRNA synthesis, or \textit{in vitro}. In intact cells three electrophoretically distinct virus-specific proteins were synthesized, with apparent molecular weights of approximately 180000 (A), 190000 (B) and 200000 (C). The accumulation of B and C was prevented by the proline analogue azetidine. Only protein A was detected \textit{in vitro}. Proteins B and C were not detected in normally infected cells. All three were associated with the nuclear fraction of cell homogenates and A and B were phosphorylated. The radioactivity of B and C declined during a chase period while that of A increased. This change was prevented by adding cycloheximide during the chase. The pattern of chymotrypsin digestion products suggested that A and B at least were similar proteins. It is presumed that protein A is the single immediate-early protein previously described and analogous to ICP 4 of herpes simplex virus. The significance and function, if any, of proteins B and C is not known but it is possible that they represent stages in the formation or transport of A within the cell and that the progression depends on an unstable protein which is depleted in cells treated with cycloheximide.

INTRODUCTION

The \( \alpha \) or immediate-early proteins of herpes simplex virus (HSV) are those whose mRNA is transcribed soon after infection in the absence of prior protein synthesis. When the inhibitor is removed and actinomycin is added to prevent any further RNA synthesis the \( \alpha \) mRNAs are translated, producing \( \alpha \) polypeptides (Honess & Roizman, 1974). The appearance of \( \alpha \) proteins leads to the production of \( \beta \) proteins which in turn suppress the synthesis of \( \alpha \) proteins. If the proline analogue azetidine is added, abnormally large amounts of \( \alpha \) proteins are synthesized; presumably the protein responsible for switching off \( \alpha \) protein production is either not made or is defective (Honess & Roizman, 1975). Five \( \alpha \) polypeptides of HSV-1 have been detected: ICP 4 (otherwise designated IE 175), ICP 0 (IE 110), ICP 22 (IE 68), ICP 27 (IE 63) and ICP 47 (IE 12). Of these, only ICP 4 has been shown to be essential in initiating and maintaining the production of later virus proteins (Preston, 1979; Watson & Clements, 1980; Dixon & Schaffer, 1980; Preston, 1981). ICP 22 is not essential for growth of the virus in Vero cells (Post & Roizman, 1981).

In cells infected with pseudorabies virus (PRV) in the presence of cycloheximide, immediate-early proteins were synthesized after removal of the inhibitor and accumulated in the nucleus (Rakusanova \textit{et al.}, 1971; Ben-Porat \textit{et al.}, 1975). A single immediate-early region of the genome was transcribed in the presence of cycloheximide (Feldman \textit{et al.}, 1979, 1982), yielding a single mRNA which was translated into a protein of mol. wt. about 180000 (180K) on reversal of the inhibition (Ihara \textit{et al.}, 1983). Studies with a temperature-sensitive mutant showed that this protein has a function similar to that of ICP 4 (IE 175) of HSV-1 (Ihara \textit{et al.}, 1983).
In a comparative study using type 1 and type 2 strains of HSV in Vero cells we found that although functional mRNA for ICP 4 of both HSV-1(F) and HSV-2(G) accumulated in the presence of azetidine, the accumulation of the HSV type 2 mRNA was prevented by adding cycloheximide at the time of infection whereas that of HSV type 1 mRNA was unaffected (Fenwick & Clark, 1982, 1983). It was suggested that a protein made soon after infection is needed to stabilize the α ICP 4 mRNA of HSV-2. In similar experiments with PRV, reported here, we have observed that it resembles HSV-1 in that functional mRNA for the immediately-early 180K protein accumulates in the presence of azetidine and the accumulation is little affected by cycloheximide. However, after reversal of cycloheximide inhibition in cells infected with PRV, the synthesis of three electrophoretically distinct proteins was detected, two of which have no obvious counterparts in HSV-infected cells.

METHODS

Pseudorabies virus (Kaplan & Vatter, 1959) was obtained from W. S. Stevely, Glasgow University, and grown from a low multiplicity inoculum in African green monkey kidney (Vero) cells. Infected cells were disrupted by ultrasonication and whole lysates (containing about 10⁹ p.f.u./ml) stored at −70°C.

Confluent monolayers of Vero cells in 25 cm² tissue culture flasks were 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.

Electrophoresis of proteins in SDS-polyacrylamide gradient gels has been described previously (Fenwick et al., 1978). Labelling with ¹⁴C-protein hydrolysate or ³²P⁻ was carried out in medium lacking amino acids or phosphate respectively and in the presence of 2 μg/ml actinomycin D unless otherwise stated. Extraction and translation in vitro of cytoplasmic RNA was described by Fenwick & Clark (1982).

Peptide mapping was done according to Cleveland et al. (1977). The first gradient gel was lightly stained and destained, appropriate sections were cut out, inserted into the wells of a second gel (15% acrylamide) and overlaid with 10 μl chymotrypsin solution (1 mg/ml) just before starting the electrophoresis.

RESULTS

Cycloheximide reversal

Cells were infected with PRV and proteins were labelled at intervals and examined by electrophoresis and autoradiography. Fig. 1 shows the developing pattern of synthesis of virus-specific proteins together with the proteins obtained from cells infected with HSV-1(F) or with HSV-2(G) for comparison. ICP 4 of HSV-1(F) (apparent mol. wt. approximately 175000) and HSV-2(G) (180000) can be seen. (These proteins are modified after synthesis and form multiple bands in the gel depending on the conditions of labelling and electrophoresis.) A PRV protein migrated at about the same rate as ICP 4 of HSV-2(G). Its rate of synthesis had begun to decline by 6 to 7 h, by which time the synthesis of cellular proteins had fallen to a low level. Other cells were infected with PRV and incubated in the presence of cycloheximide and then labelled after removing the inhibitor in order to distinguish α polypeptides. Three distinct bands of radioactive protein were seen in the autoradiogram, two heavily labelled with apparent mol. wt. of approximately 180000 and 190000 and one lightly labelled at about 200000, marked A, B and C respectively in Fig. 1. Host protein synthesis remained unimpaired. All three of these proteins were associated predominantly with the nuclear fraction of a cell homogenate rather than with the cytoplasmic fraction (data not shown) as is the case with α polypeptides of HSV-1 and HSV-2 (Pereira et al., 1977; Fenwick et al., 1978).

Pulsing and chasing

In order to reveal possible precursor-product relationships between the proteins A, B and C they were examined after a 30 min pulse of ¹⁴C-amino acids or after a subsequent chase period of 1 h in non-radioactive medium in the presence of cycloheximide to prevent further incorporation of label. There was no change in the distribution of radioactivity between the three bands during the chase (Fig. 2) but each migrated slightly more slowly after chasing, suggesting a post-translational modification similar to those of ICP 4 of HSV (Pereira et al.,
Protein A at least can be seen to consist of two bands, the lower of which disappeared during the chase. The intensities of bands A, B and C were measured on autoradiograms after 20 min and 60 min labelling periods (Fig. 3a) and after a chase in the presence or absence of cycloheximide (Fig. 3b, c). The radioactivity of proteins A and B increased in parallel between 20 min and 60 min but that of C had reached a steady state within 20 min. There was again little change in the distribution of radioactivity during a chase in the presence of cycloheximide (Fig. 3b) but without cycloheximide the intensities of bands B and C declined while that of A increased (Fig. 3c). Evidently protein A was not being converted to B or C during the course of these experiments. While proteins A and B appeared to be made in parallel, B was slowly processed or degraded, possibly yielding A. C was also turning over, probably more rapidly. The decline of both B and C was arrested if cycloheximide was added.

**Effect of azetidine**

As explained earlier, the initial object of this study was to examine the effect of cycloheximide on the accumulation of PRV α mRNA in order to compare it with HSV. It is clear from Fig. 1 that α mRNA was readily detectable after several hours in the presence of cycloheximide. When cells were treated with azetidine from the start of infection and labelled from 4 to 5 h, the synthesis of a protein migrating slightly faster than the 180K protein of normally infected cells was greatly enhanced (Fig. 4, compare lanes 1 and 3) just as has been found with HSV-1 and HSV-2. If cycloheximide was also added during the same period and then removed before labelling, the synthesis of this protein was only slightly reduced (compare lanes 3 and 4). In this respect, therefore, PRV resembled several strains of HSV-1 and differed from HSV-2(G) (Fenwick & Clark, 1983). The absence of proteins resembling B and C from lysates of cells...
Fig. 2. Modification of proteins A, B and C. Cells were labelled for 30 min after reversal of a cycloheximide block at 3 h after infection. Some were lysed (P) while others were washed and incubated for a further 1 h in the presence of cycloheximide (Ch).

Fig. 3. Changes in distribution of label during pulse and chase. Cells were labelled after removing cycloheximide at 3 h after infection. After electrophoresis of equal aliquots of lysates, the regions of the autoradiogram corresponding to proteins A, B and C were scanned with a densitometer. (a) 20 min (lower curve) and 60 min (upper curve) labelling periods; (b) 60 min label (broken curve) followed by 2 h chase (solid curve) in the presence of cycloheximide; (c) 60 min label (broken curve) followed by 2 h chase (solid curve) in non-radioactive medium without cycloheximide.

treated with both azetidine and cycloheximide together (lane 4) as compared to those treated with cycloheximide alone (lane 5) was presumably due to residual azetidine remaining after washing the cells before labelling since cycloheximide, in inhibiting protein synthesis, would prevent the incorporation of azetidine. A slightly lesser effect of azetidine added with the label, after removing cycloheximide (lane 6), may reflect a small delay in the incorporation of the analogue.

Thus, proteins B and C, although clearly α proteins by the cycloheximide reversal test, unlike the α proteins of HSV did not accumulate in the presence of azetidine. This suggests that proteins A, B and C may be interrelated and that if made in the presence of azetidine either the conversion from A to B and C is prevented or the conversion to A is accelerated.

In further attempts to characterize the three α proteins we examined their phosphorylation, their synthesis in vitro and their breakdown by chymotrypsin. Proteins A and B were phosphorylated either during or soon after synthesis. Cells were labelled with either $^{32}$P$_i$ or $^{14}$C-amino acids 3 h after infection in the presence of either cycloheximide or azetidine. The distributions of the two labels in bands A and B of the autoradiogram were closely similar (data not shown). Protein C was lightly labelled with $^{14}$C but a corresponding $^{32}$P-labelle band could not be detected on the autoradiogram.

**In vitro translation**

If the proteins A, B and C are unrelated, their mRNAs must be present in infected cells that have been incubated in the presence of cycloheximide. Attempts to detect them by translation
**Fig. 4.** Effect of azetidine on the formation of proteins A, B and C. Infected cells were treated with azetidine (AZ) or cycloheximide (CX) during the intervals shown. All were labelled 4 to 5 h.

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Proteins were therefore made and Fig. 5 shows the translation products. Proteins were also labelled in duplicate cultures of intact cells for comparison. There is a strong resemblance between the patterns of radioactive proteins labelled *in vivo* and *in vitro*, but among the differences is the absence of bands B and C from the labelled sample *in vitro* (compare lanes 4 and 8). No functional mRNA for B or C was detected although mRNA for A was present in cells infected in the presence of cycloheximide or azetidine (lanes 4 and 5). On the other hand the mRNA for A that was present in untreated infected cells was not detected *in vitro* (compare lanes 3 and 7). Thus, there may be some selectivity in the survival or translation of different mRNAs *in vitro*.

**Proteolysis**

Pieces of gel containing labelled proteins A, B and C were cut out and applied to a second gel together with chymotrypsin and again subjected to electrophoresis and autoradiography. The patterns of degradation products of A and B were apparently the same (Fig. 6). That of C was not reliably discernible. We can conclude that A and B are probably different forms of the same protein or related proteins.

**Discussion**

In Vero cells infected with PRV in the presence of cycloheximide three electrophoretically distinct α proteins (A, 180K; B, 190K; C, 200K) were made after removing the inhibitor. All three proteins were modified to slightly slower-migrating forms during a chase period in the presence of cycloheximide, as are a number of HSV proteins, including the α proteins. Like HSV
Fig. 5. Translation of RNA in vitro. Duplicate cultures of infected cells were incubated with or without cycloheximide (CX) or azetidine (AZ) as indicated for 4 h. Cytoplasmic RNA was extracted from one culture of each pair and translated in vitro in the presence of [35S]methionine (lanes 1 to 5). B is a control translation without added RNA. The other cultures were washed and labelled for 30 min with 14C-amino acids (lanes 6 to 9). A 9% polyacrylamide gel was used for the electrophoresis.

x proteins, they accumulated in the nuclear fraction of cell homogenates and A and B, at least, were phosphorylated. Protein A probably corresponds to ICP 4 of HSV and to the single immediate-early 180K protein described by Ihara et al. (1983) in primary rabbit kidney cell cultures infected with PRV. Proteins B and C were also observed after cycloheximide reversal in BHK cells (M. L. Fenwick & M. McMenamin, unpublished experiments) but have no obvious counterparts in HSV-infected cells. They were not seen in rabbit kidney cells (Ihara et al., 1983), perhaps because of some property of the cells or because cycloheximide was added 1 h before infection, possibly resulting in the loss of some unstable cellular component. In our experiments cycloheximide was added immediately after infection in order to be comparable to our previous work with HSV-1 and HSV-2. The rabbit kidney cell system seems to differ in several respects from the Vero cell system used here. In Vero cells, synthesis of the 180K protein is readily detectable by labelling and electrophoresis up to 6 h after infection (Fig. 1). Host cell protein synthesis is not suppressed during the first hour after reversing a cycloheximide block (Fig. 1) and expression of later virus genes ensues if actinomycin is not added (unpublished experiments).

The interpretation of in vitro translation is subject to the reservation, mentioned in the Results section, that the absence of a band from an autoradiogram does not prove that the mRNA for that protein was not present in the cell. Nevertheless, the results are consistent with those of
Ihara et al. (1983), who observed a single mRNA in cycloheximide-treated cells which was translated in vitro into the 180K protein. They suggest that A is the primary translation product of the α mRNA and that B and C arise by interaction with some component of the intact cell, forming an association that survives treatment with SDS and mercaptoethanol during the preparation of the samples for electrophoresis. However, the results of labelling in vivo are not consistent with a simple precursor–product relationship between A and B or C. Comparison of the distribution of radioactivity after 20 min and 60 min labelling periods suggests that a relatively small pool of C turns over rapidly while A and B accumulate in parallel, and during a non-radioactive chase the amount of label in B and C declined while that in A increased. If cycloheximide was present during the chase, there was little change in the distribution of label, implying that protein synthesis is required for the decline of B and C to occur, possibly the synthesis of an unstable protein that was lost during the initial incubation with cycloheximide. The existence of such a protein might account for the failure to detect B or C in normally infected cells if they were quickly converted to A. The accumulation of proteins B and C was prevented by azetidine. It is possible that incorporation of azetidine into protein A would prevent its association with a postulated cellular component.

Thus a provisional explanation of the observed phenomena is that the single α mRNA is translated into protein A (180K) which forms an early transient attachment to a cellular component to form protein C (200K). Protein C is degraded, possibly yielding B (190K) and A. The significance and function, if any, of proteins B and C is unknown. One possibility is that they might be intermediates in the transport of the active protein A to the nucleus.
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REFERENCES


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