Vaccinia Virus Polypeptide Synthesis: sequential Appearance and Stability of Pre- and Post-replicative Polypeptides

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SUMMARY

The polypeptides of BSC1 cells infected with vaccinia virus and pulse labelled with [$^{14}$C]-protein hydrolysate or [$^{35}$S]-methionine have been examined by discontinuous polyacrylamide gel electrophoresis followed by autoradiography. About 80 virus induced polypeptides were detected, 30 appearing before and 50 after the onset of virus DNA synthesis. These were termed pre- and post-replicative polypeptides respectively. The synthesis of most pre-replicative polypeptides started shortly after the peak of virus DNA synthesis; when virus DNA synthesis was inhibited this turn-off did not occur and post-replicative polypeptides were not made. The synthesis of some post-replicative polypeptides started at about the time of maximal DNA synthesis, reached a peak about 1 h later, and was then turned off. Other post-replicative polypeptides whose synthesis started at this time were made for prolonged periods. The synthesis of most post-replicative polypeptides started about 1 h after the time of maximal virus DNA synthesis and continued thereafter for prolonged periods. The stability of pre- and post-replicative polypeptides was examined in pulse-chase experiments; most pre-replicative and ‘early’ post-replicative polypeptides were stable for prolonged periods, whereas, during a chase, eleven ‘late’ post-replicative polypeptides disappeared and seven new polypeptides appeared.

INTRODUCTION

Numerous investigations have shown that vaccinia virus-induced proteins can be divided into ‘early’ and ‘late’ classes, the former being synthesized before, and the latter after, virus DNA synthesis (reviewed by Joklik, 1968; Moss & Salzman, 1968; Esteban & Metz, 1973). Included in the ‘early’ category are several enzymes concerned with nucleic acid metabolism (McAuslan, 1963; Jungwirth & Joklik, 1965; McAuslan & Kates, 1967) and some structural proteins of the virus particle (Holowczak & Joklik, 1967). The majority of virus particle structural proteins are synthesized after the onset of virus DNA synthesis (Holowczak & Joklik, 1967).

The rapid cessation of host protein synthesis which follows infection with high input multiplicities of virus (Moss, 1968) permits the ready identification of virus-induced proteins in unfractionated extracts of cells labelled with radioactive amino acids at various times after infection.

This paper reports the results of studies on vaccinia virus polypeptide synthesis in BSC1 cell monolayers using high resolution SDS-polyacrylamide gel electrophoresis followed by autoradiography.
METHODS

Materials. [¹⁴C]-uridine protein hydrolysate (57 mCi/matom), [³⁵S]-l-methionine (175 Ci/mmol) and [methyl-³H]-thymidine (18·4 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. Hydroxyurea and cytosine arabinoside (cytosine-1-β-d-arabinofuranosyl-HCl) were purchased from the Sigma Chemical Co., St Louis, Missouri, U.S.A.

Virus, cells and infection. Vaccinia virus (Evans vaccine strain) was grown and titrated in monolayers of BHK21 cells, and purified by the method of Joklik (1962). Monolayers of cultures of BSC1 cells were grown in Eagle’s medium (Glasgow modification) containing 10% foetal bovine serum and were infected with purified virus at an input multiplicity of 50 p.f.u./cell in Eagle’s medium containing 2% foetal bovine serum and 0·02 M-MgCl₂. Absorption was allowed to continue for 30 min at 38 °C. The monolayers were then washed and maintained in Eagle’s medium containing 5% foetal bovine serum.

Labelling of proteins with [¹⁴C]-labelled amino acids and [³⁵S]-methionine. Cell monolayers were washed twice with warm amino acid-free Eagle’s medium (before labelling with [¹⁴C]-labelled amino acids) or with warm methionine-free Eagle’s medium (before labelling with [³⁵S]-methionine). Proteins were then labelled by the addition of [¹⁴C]-protein hydrolysate (5 μCi/ml) contained in amino acid-free Eagle’s medium or with [³⁵S]-methionine (50 μCi/ml) contained in methionine-free Eagle’s medium. After 15 min of incubation at 38 °C, the medium was removed and the cells from each plate were scraped into 0·4 ml of 0·001 M-tris-hydrochloride, pH 9·0. Samples were stored at −70 °C prior to electrophoresis.

Pulse-chase experiments. Infected cell monolayers were labelled with [³⁵S]-methionine as described above. At the end of the 15 min labelling period the label was removed and the cells were washed and incubated further in Eagle’s medium containing 100 times the normal amount of methionine. At appropriate times the cells were scraped into 0·001 M-tris-hydrochloride, pH 9·0 and analysed by electrophoresis.

Gel electrophoresis and autoradiography. Samples were reduced and dissociated by boiling for 90 s in 1% mercaptoethanol and 2% SDS. After the addition of sucrose and tracking dye the samples were subjected to electrophoresis in 9% or 12% acrylamide gels (10 cm long) using the discontinuous SDS buffer system described by Laemmli (1970). After electrophoresis the gels were stained with Coomassie brilliant blue, de-stained and sliced longitudinally using the apparatus described by Fairbanks, Levinthal & Reeder (1965). The central slices were then placed on Whatman 3 MM paper and aligned using the stained bands, which were largely of BSC1 cell origin, as markers. The slices were dried using a procedure based on that of Fairbanks et al. (1965). Autoradiography was done with Kodirex KD 54T X-ray film. Polypeptide mol. wt. were determined using phospholipase A, catalase, fumarase, glyceraldehyde dehydrogenase and chymotrypsin A for calibration.

DNA synthesis in infected cells: effect of inhibitors. Cells were maintained after infection in the presence or absence of cytosine arabinoside or hydroxyurea and were labelled at 2 h post-infection (p.i.) with 10 μCi of [³H]-thymidine per ml for 15 min. Each monolayer was harvested into 0·5 ml of ice-cold 0·002 M-tris-hydrochloride, pH 9·0, and 0·05 ml samples were applied to filter-paper discs which were washed with cold trichloroacetic acid prior to determination of radioactivity.
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RESULTS

Time course of polypeptide synthesis in infected cells

The time course of polypeptide synthesis in infected cells was followed in experiments where infected cells were pulse labelled for 15 min with $^{[14]C}$-amino acids or $^{[35]S}$-methionine at various times after infection. Samples were then analysed by polyacrylamide gel electrophoresis and autoradiography. The results of typical experiments are shown in Fig. 1(a) and 2, and are summarized in schematic form in Fig. 3. Comparison of autoradiographs obtained with $^{[14]C}$-amino acids and $^{[35]S}$-methionine revealed no major differences in labelling patterns, justifying the use of $^{[35]S}$-methionine as a general protein label in subsequent experiments.

The autoradiographs showed a complex pattern of bands which was, however, highly reproducible from experiment to experiment. A small number of polypeptides not present in uninfected cell samples could be detected in samples labelled at the end of the virus absorption period, i.e. from 30 to 45 min after the addition of virus to the cell monolayers. With only one exception the rate of synthesis of these early virus polypeptides increased, judged by band intensity, as the virus growth cycle progressed. The rate of synthesis of many early polypeptides reached a maximum at about 2 h after infection and then declined. One polypeptide (mol. wt. slightly greater than 74000) was only detected in samples labelled up to 90 min after infection.

The rate of synthesis of a number of early polypeptides declined rapidly to zero after 3 h p.i. This pattern of synthesis was clearly shown, for example, by a polypeptide with a mol. wt. of 25000 which was synthesized in large amounts throughout this early period. Some...
Fig. 2. Autoradiograph showing time course of polypeptide synthesis in infected cells. At the times indicated (h p.i.) the cells were labelled for 15 min with [35S]-methionine. Samples were electrophoresed in 12 % acrylamide gels. U, uninfected cells.

other early polypeptides were made throughout the virus growth cycle, although they were synthesized in reduced amounts at late times, i.e. after 3 to 4 h p.i. This pattern of synthesis was shown by three polypeptides, each with a mol. wt. greater than 100000. The synthesis of these polypeptides started between 60 and 90 min after infection, somewhat later than the start of synthesis of most of the other early polypeptides which were first detected between 30 and 60 min p.i.

The synthesis of several polypeptides started at between 2 and 2½ h after infection. The subsequent pattern of synthesis of these polypeptides varied; two prominent members of this group, with mol. wt. of about 34000 and 28000, were made in maximal amounts at 3 h p.i., their rate of synthesis then declining rapidly, whereas another major polypeptide, mol. wt. 65000, achieved its maximal rate of synthesis at 3 h p.i. and maintained this rate for many hours.

A large number of polypeptides were first detected in samples labelled between 3 and 4 h
Vaccinia virus polypeptide synthesis

Fig. 3. Schematic diagram of virus-induced polypeptides showing time course of appearance, mol. wt., and the position of two major virus particle polypeptide precursors, P4a and P4b.

The rate of synthesis of these late polypeptides did not appear to vary significantly between 4 and 10 h p.i.

A total of about 80 new polypeptides were detected in autoradiographs of gels analysing infected cell preparations, 30 appearing in the early part of the virus growth cycle and about 50 at late times. Host protein synthesis declined after the addition of virus and was completely shut off by 3 h p.i. It is likely that the number of early polypeptides quoted here is an underestimate due to the masking of new virus polypeptides by residual host synthesis.
Identification of pre- and post-replicative polypeptides

The proteins synthesised in cells infected with vaccinia virus can be divided into two classes, early proteins being made before and late proteins after virus DNA synthesis (Joklik, 1968). As the results reported in this paper show that more than two classes of virus proteins can be distinguished on a temporal basis, the terms 'early' and 'late' are replaced here by 'pre-' and 'post-replicative', using the latter terms to indicate virus proteins made before and after virus DNA synthesis, respectively. This nomenclature has previously been used to describe similar classes of bacteriophage T4 proteins (O'Farrell & Gold, 1973).

Vaccinia pre- and post-replicative polypeptides were identified in this study using cytosine arabinoside (CAR) as an inhibitor of virus DNA synthesis (Fig. 1b). The concentration of drug used in these experiments (25 μg/ml) inhibited DNA synthesis in infected cells by 94%. No difference was detected between the polypeptide profiles of CAR treated and control samples taken up to 2 h after infection. After this time, however, no new polypeptides appeared in CAR treated cells, and a polypeptide profile grossly similar to the 2 h pattern was seen with samples taken up to 8 h after infection, the switch-off which normally occurs in the synthesis of many pre-replicative polypeptides failing to occur. The switch-off of host polypeptide synthesis which occurred under normal condition after 3 h p.i. was also less marked in the CAR treated samples.

These results allow post-replicative polypeptides to be identified as those which appear for the first time at or after 2 h p.i. The onset of post-replicative polypeptide synthesis thus coincides with the peak of virus DNA synthesis, which occurs at 2 h p.i. in this system (Pennington & Follett, 1974).

Sequential appearance of post-replicative polypeptides

The time course studies reported above indicate that at least two classes of post-replicative polypeptides can be defined on a temporal basis, the onset of synthesis of some polypeptides occurring between 2 and 2½ h p.i. and the synthesis of others starting between 3 and 4 h p.i. Confirmation of these findings were obtained in experiments which followed the appearance of post-replicative polypeptides after the removal of a reversible inhibitor of virus DNA synthesis. Cells were infected and maintained in medium containing 10⁻³ M-hydroxyurea. This concentration of drug-inhibited virus DNA synthesis by 97%. At 4 h p.i. the hydroxyurea was removed and the monolayers were maintained in normal medium for a further 3 h. Autoradiographs showing polypeptides labelled at various times during this experiment are shown in Fig. 4. Post-replicative polypeptides were first detected in samples labelled between 1 and 1½ h after the removal of hydroxyurea. The subsequent turning-on of synthesis of other polypeptides in this class occurred sequentially with a relative timing identical to that seen during the course of a normal infection (compare Fig. 4 with Fig. 1(a) and 2). The termination of synthesis of some pre- and post-replicative polypeptides which was observed in normal infections was also observed when hydroxyurea was removed, the relationship between these events and the onset of synthesis of the various classes of post-replicative polypeptides being similar to that observed in normal infections.

Pulse-chase experiments; stability and cleavage of pre- and post-replicative polypeptides

The stability of pre- and post-replicative polypeptides was examined by analysis of polypeptides in samples from pulse-chase experiments initiated at various time during the virus growth cycle. The fate of pre-replicative and 'early' post-replicative polypeptides was followed after pulse-labelling infected cells between 2½ and 2¾ h after infection. The
majority of polypeptides labelled at this time appeared to be stable throughout the chase period (up to 9 h after the removal of labelled amino acid) (Fig. 5). However, a number of bands disappeared from samples taken during the first hour of the chase period. In addition, one new band appeared in samples taken during the chase period; it was first detected after a 60 min chase and persisted thereafter in unchanged amounts until the termination of the chase.

To examine the fate of 'late' post-replicative polypeptides, label was added between 6 and 6½ h p.i. and samples were taken at various times during a 17 h chase period. Autoradiograms of gels analysing these samples are shown in Fig. 6. During the chase period 11 bands disappeared or became significantly reduced in intensity during the chase period and 7 new bands appeared. Some of these changes have been previously shown to be due to cleavage of virus particle structural protein precursors, polypeptides P4a and P4b being
processed to form the major core proteins 4a and 4b (Moss & Rosenblum, 1973). The results presented here indicate that it is likely that other cleavages occur late in the virus growth cycle, but further work will be required to establish precursor-product relationships. Visual inspection of autoradiograms led to the conclusion that the rates of disappearance of different polypeptides varied considerably; this finding was confirmed by quantitative densitometry (Fig. 7). Work is in progress to assess the significance of this finding.

**DISCUSSION**

Previous studies on the kinetics of protein synthesis in cells infected with vaccinia virus have used enzymological, immunological and gel electrophoretic techniques (Joklik, 1968; Moss & Salzman, 1968; Esteban & Metz, 1973) and they have revealed an overall pattern of synthesis similar to that demonstrated in this study. Host protein synthesis is rapidly turned-off after infection, and the synthesis of new proteins is detectable at very early times in the virus growth cycle. The onset of synthesis of many virus proteins is dependent, however, on prior virus DNA synthesis; recent studies employing tryptic peptide analyses
have confirmed that some of the proteins in this group are structural components of the virus particle (Moss & Rosenblum, 1973). It is known that most of the virus particle structural proteins are synthesized late in infection (Holowczak & Joklik, 1967). Shortly after the onset of post-replicative protein synthesis the synthesis of many, but not all, of the pre-replicative proteins is terminated. Strong evidence that the new proteins which appear after infection are virus coded has been provided by the recent demonstration that the pattern of virus polypeptide synthesis in enucleate cells is qualitatively indistinguishable from that in normal cells (Pennington & Follett, 1974).

The vaccinia virus genome is large enough to code for between 200 and 400 average-sized proteins, the coding capacity expressed in total mol. wt. of protein being between $8 \times 10^6$
Fig. 7. Kinetics of disappearance of post-replicative polypeptides during a pulse-chase experiment. Densitometer tracings were prepared from the autoradiograph shown in Fig. 6 using a Joyce-Loebl densitometer and peak heights were measured. The density of a stable polypeptide, NS3, was used to normalize the densities of polypeptides in different gels. ●, P4a; ○, P4b; ■, Y; □, Z.

and $16 \times 10^6$. About 80 virus-coded polypeptides, with a total mol. wt. of $3 \times 10^6$, were demonstrated in the present study. It is likely that some virus polypeptides are synthesised in amounts too small to be detected by the methods used here; in addition some polypeptides probably escaped detection due to their masking by residual host protein synthesis early in infection and due to the co-migration of different virus polypeptides which have similar or identical mol. wt. After taking these factors into account, however, the discrepancy between the potential coding capacity and the observed number of polypeptides still remains large and the possibility that a significant proportion of the vaccinia virus genome does not code for polypeptides cannot be ruled out.

The regulation of pre-replicative protein synthesis has been discussed in detail by Esteban & Metz (1973). The use by these workers of a different system (L cells infected with vaccinia virus strain WR) and a different gel electrophoresis technique introduces uncertainties when a direct comparison of their findings is made with the results of the present study. One major difference revealed by this comparison, however, is that in the L cell system two polypeptides, with mol. wt. of 60000 and 27000 respectively, were made in large amounts immediately after infection, their synthesis then terminating at about 60 min p.i. (Esteban & Metz, 1973). These polypeptides were not detected in the present study, although a polypeptide with a mol. wt. of about 74000 was detected only in samples labelled during the first 90 min of infection. This polypeptide was made in small amounts. Studies are in progress in this laboratory to establish the pattern of synthesis of different pre-replicative proteins before and after the uncoating of the virus core.

The sequential appearance of polypeptides whose synthesis is dependent on virus DNA
Vaccinia virus polypeptide synthesis has previously been observed by Moss & Salzman (1968). These workers showed that the onset of synthesis of the majority of post-replicative polypeptides was preceded by the appearance of another, single, post-replicative polypeptide. The rate of synthesis of this polypeptide declined during the later part of infection. The results reported here were obtained using a gel system with higher resolution than that used by Moss & Salzman and show that the post-replicative polypeptides can be divided into at least two groups on a temporal basis, the synthesis of the 'early' group starting at about the time of maximal DNA synthesis. The rate of synthesis of some of the polypeptides in this group declined rapidly after reaching a peak at about 3 h p.i., whereas another member of this group, mol. wt. 65,000, continued to be made in relatively large amounts until very late times. The possible role of this polypeptide in immature virus particle formation has been discussed elsewhere (Pennington, 1973).

The sequential appearance of various classes of post-replicative proteins appears to be a phenomenon restricted to pox viruses, other large DNA viruses such as bacteriophage T4 and adenoviruses not exhibiting this phenomenon (Russell & Skehel, 1972; O'Farrell & Gold, 1973).

The stability of pre- and post-replicative polypeptides was followed by pulse chase experiments. These showed that the majority of pre-replicative and 'early' post-replicative polypeptides were stable for many hours. A small number of polypeptides disappeared rapidly during the chase period, however; the possibility that these polypeptides were either rapidly lost from the cells into the medium or underwent proteolysis was not further investigated. One new band appeared in autoradiograms of chase samples, suggesting that at least one of these polypeptides is processed by cleavage.

In contrast, many post-replicative polypeptides appeared to be unstable, at least 11 bands disappearing from autoradiograms of samples taken during the chase period. As already indicated, some of these changes have been shown to be due to the cleavage of polypeptides to form structural components of the virus particle. Whether all the changes observed during chase periods can be accounted for by this mechanism or whether some post-replicative polypeptides are destroyed after a brief existence or are lost from the cell into the medium remains to be elucidated.

Little is known about the control mechanisms responsible for the sequential appearance of the various groups of pre- and post-replicative proteins, the selective switching-off of their synthesis at various times during the virus growth cycle, and the maintenance of the various rates of synthesis observed with different polypeptides, although it seems clear that some controls are exerted at the transcriptional level (reviewed in Joklik, 1968). The function of the polypeptides described in this study also remain to be elucidated, the complexity of gel profiles and of the changes occurring during chase periods late in the virus growth cycle rendering the identification of virus particle structural polypeptides a far from straightforward task.

REFERENCES


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