Vaccinia Virus Morphogenesis: a Comparison of Virus-induced Antigens and Polypeptides

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SUMMARY

Vaccinia virus-induced antigens and polypeptides labelled late in infection with [14C]-leucine were examined in gel precipitin tests and polyacrylamide gel electrophoresis followed by autoradiography. Pulse-chase experiments, investigations on the effects of rifampicin and direct analysis of precipitin lines by gel electrophoresis allowed the identification of two major precipitin lines in terms of the polypeptides specific for the infected cell. One of these was non-structural. A small proportion of the other antigen was incorporated into sedimentable structures during a chase; this was prevented by rifampicin. Evidence suggesting that three virus-specified polypeptides are formed by cleavage of larger precursors is also presented and discussed.

INTRODUCTION

Many investigators have studied the soluble antigens produced during the growth of vaccinia virus. Some of these antigens have been demonstrated in virus particles (Marquardt, Holm & Lycke, 1965; Westwood et al. 1965; Cohen & Wilcox, 1968) but the function of the remainder is unclear. Recent work on the polypeptides synthesized during infection has shown that virus maturation is a complex process, at least one major structural polypeptide being formed by the cleavage of a larger precursor molecule (Katz & Moss, 1970a). Rifampicin, a specific and reversible inhibitor of vaccinia virus maturation (Moss et al. 1969; Pennington, Follett & Szilagyi, 1970) blocks this cleavage and prevents the integration of another polypeptide into a particulate fraction of the cytoplasm (Katz & Moss, 1970b).

This paper reports experiments which compared virus-induced antigens and polypeptides synthesized late in infection. The object of these experiments was to identify soluble antigens in terms of virus-induced polypeptides and, using this combined immunological and biochemical approach, to gain further information concerning virus assembly.

METHODS

Materials. Rifampicin was a gift from Professor P. Sensi and colleagues of Lepetit S.P.A. (Milan) and Lepetit Pharmaceuticals Ltd., Slough, Buckinghamshire. Uniformly labelled [14C]-L-leucine of specific activity 331 mCi/m-mol was purchased from the Radiochemical Centre, Amersham, Buckinghamshire.

Infection of cells and labelling with [14C]-leucine. Monolayer cultures of BHK 21 cells (clone 13) were infected with vaccinia virus (EVANS vaccine strain) at an input multiplicity of 50 p.f.u./cell. After absorption for 1 h at 38 °C, the cells were washed and, after the addition of Eagle's medium (Glasgow modification) containing 5 % calf serum, incubation
was continued at 38 °C. At the time of labelling the medium was removed and replaced with warm medium containing 5 µCi/ml [14C]-leucine and one-tenth the normal amount of amino acids. In pulse-chase experiments labelling was terminated by the removal of label and the addition of medium containing 100 times the normal amount of leucine and 10% calf serum. In experiments where label was present during the whole infectious cycle the medium contained the normal amount of amino acids and 2% calf serum.

**Antiserum.** Rabbitpox antiserum was a gift from Dr G. Appleyard. It was a pool obtained from rabbits surviving infection with rabbitpox virus and which had subsequently been given from 4 to 6 intravenous doses of 10⁸ to 10⁹ p.f.u. of rabbitpox virus grown in rabbit testis.

**Preparation of samples for immunodiffusion and polyacrylamide gel electrophoresis.** Cells were washed with ice-cold Eagle's medium and allowed to swell in cold 1 mM-tris-HCl (pH 9.0) for about 30 min. After disruption, using a glass homogenizer with a tightly fitting Teflon plunger, cell debris and nuclei were removed by centrifuging at 225 g for 5 min. Some preparations were layered on to 20% sucrose (v/v) in 0.001 M-tris-HCl and separated into sedimentable and non-sedimentable fractions by centrifuging at 18000 g for 1 h. This procedure allowed the convenient handling of small volumes and ensured that no contamination of pellet material with supernatant occurred during sample collection. Pellets were resuspended in a volume of 0.001 M-tris-HCl equivalent to that of the supernatant fluid at the end of the centrifuging.

**NP40 treatment of samples.** NP40 was added to a final concentration of 0.5% and samples were incubated at 37 °C for 30 min.

**Gel precipitin autoradiography.** Gel precipitin tests were done in 1% Noble's agar cast on microscope slides. The distance between the centres of the central and the six peripheral wells was 9 mm and the well diameter was 4 mm. Radioactive samples were mixed with an equal volume of cell homogenate, prepared 40 h after infection, which contained large amounts of vaccinia-induced soluble antigens. This acted as carrier and ensured a reproducible precipitin band pattern in all tests, allowing interpretation of autoradiographs to be semi-quantitative. Radioactive samples to be compared were placed in opposing wells; the remaining four wells were filled with the unlabelled antigen preparation described above and the central well was filled with antiserum. Diffusion was allowed to take place for 2 days at room temperature. The gels were then washed for 2 days in phosphate buffered saline (pH 7.4) containing 1% (w/v) sodium azide, followed by a 2 h rinse in distilled water. The gels were dried overnight at 37 °C.

 Autoradiography was done using the fast side of Kodak Radiation Monitoring film. After autoradiography gels were stained with amido black and radioactive precipitin lines were identified by superimposing the autoradiograph over the stained gel.

**Polyacrylamide gel electrophoresis.** 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 10 cm long 7.5% acrylamide gels containing 0.1% SDS and 0.1 M-phosphate buffer, pH 7.4, at 3 mA/gel for about 17 h. Electrophoresis of some samples was done in 10% acrylamide gels using the discontinuous SDS buffer system described by Laemmli (1970). After electrophoresis, gels were washed for 1 h in 7% acetic acid (overnight in the case of discontinuous gels) and sliced and dried on to sheets of cellulose acetate according to the method of Fairbanks, Levinthal & Reeder (1965). Autoradiography was done using Kodak Royal Blue X-ray film.
RESULTS

Gel precipitin tests

A complex but reproducible pattern of precipitin lines was obtained in tests of cytoplasmic extracts prepared from cells infected for 48 h. The pattern was dominated by two major lines, designated A and B (Fig. 1), situated approximately midway between the antigen and antiserum wells. A number of weaker lines was observed close to the antiserum and antigen wells and a stronger line was seen between A and B. No precipitin lines were obtained with extracts of uninfected BHK cells.

Effect of rifampicin on antigen synthesis and distribution in subcellular fractions

The antiviral effect of rifampicin on vaccinia virus is specific and freely reversible and can be used to synchronize the production of immature virus particles (Moss et al. 1969; Pennington et al. 1970). Accordingly, experiments were done which examined the effect of rifampicin, and its removal, on the distribution of antigens A and B in subcellular fractions.

Cells were infected and maintained for 16 h in medium containing 100 μg/ml rifampicin and [14C]-leucine. The cells were harvested and the cytoplasmic fraction was separated into pellet and supernatant fluid. Supernatant fluids were examined without further treatment; pellets were treated with NP40 before testing. The autoradiographs of gel precipitin tests are illustrated in Fig. 1. Rifampicin had little effect on antigen synthesis although comparison with control samples showed that there was a reduction in the amount of label contained in the precipitin line designated V. This line was seen only in gel precipitin tests of NP40-treated pellets. Antigens A and B were both strongly labelled in the supernatant fluids from both rifampicin and control samples. A marked difference, however, was seen in the amount of antigen A solubilized from the pellets by NP40 treatment, none being present in diffusible form in the detergent-treated rifampicin sample, although this antigen was detected in the detergent-treated control sample in an amount comparable to that found in the supernatant fraction. The analysis of the other antigens solubilized from the pellets showed no significant difference between the rifampicin and control samples, with the exception of line V. No antigen B was detected in either rifampicin or control pellets.

The behaviour of antigens following the removal of rifampicin was also investigated and the results are shown in Fig. 2. Antigen A rapidly appeared in the pellet in detergent-soluble form following the removal of the antibiotic, significant amounts being detected within 10 min. In this experiment labelling of line A was maximal in the 30 min sample; thereafter the amount of label slowly declined. No changes were seen during the chase period in the amount of antigens A and B present in the supernatant fluid.

Pulse-chase experiments

The distribution of antigen A in subcellular fractions was also examined in pulse-chase experiments. Infected cells were incubated with [14C]-leucine for 15 min starting 9 h after infection. After removal of the label, the cells were then maintained for 9 h in medium containing excess unlabelled leucine. Analysis of supernatant fluids gave results similar to those of experiments examining the effects of rifampicin removal, the amount of antigen A not declining significantly during the chase period. Antigen A, however, was detected in NP40-treated pellets prepared at the end of the pulse (Fig. 3). The amount of antigen solubilized from pellets increased during the chase, reaching a maximum at 1 h after the end of the pulse. It then declined to zero.
Fig. 1. Antigens synthesized in the presence of rifampicin and their distribution in subcellular fractions. Top well: control labelled antigen preparations (no rifampicin) with equal volume of carrier antigen. Bottom well: antigens labelled in rifampicin, with equal volume of carrier antigen. Remaining wells: carrier antigen alone. (a) Supernatant fluid antigens, (b) antigens solubilized from pellet by NP_40, (c) unfractionated cytoplasmic preparations treated with NP_40. Stained slides on left, autoradiographs on right.
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Fig. 2. Effect of removing rifampicin on distribution of labelled antigens in supernatant fluids and pellets. Top well, supernatant fluids with equal volume of carrier antigen. Bottom well, NP 40-treated pellets with equal volume of carrier antigen. Remaining wells, carrier antigen alone. (a, b) Rifampicin present throughout experiment, (a) stained slide, (b) autoradiograph. (c-f) Rifampicin present for 16 h and removed for 10 min (c), 30 min (d), 1 h (e) and 3 h (f). Autoradiographs shown; the stained precipitin line patterns were identical with that of slide (a).
Fig. 3. Autoradiograph of antigens solubilized by NP40 from cytoplasmic pellets from a pulse-chase experiment. Top and bottom wells, labelled samples with an equal volume of carrier antigen. Remaining wells, carrier antigen alone. (a) Stained slide; top, end of pulse; bottom, 15 min chase. (b) Autoradiograph of (a). (c) Autoradiograph; top, 30 min chase; bottom, 1 h chase. (d) Autoradiograph; top, 3 h chase; bottom, 9 h chase. The stained precipitin line patterns of (c) and (d) were identical with that of slide (a).
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Radioactive polypeptides in vaccinia infected cells

As experiments with rifampicin and pulse-chase experiments had revealed individually characteristic changes in the distribution of antigens in cytoplasmic fractions and in their detergent solubility, virus-induced polypeptides were analysed in a similar way.

Fig. 4 shows the pattern of radioactive polypeptides in cytoplasmic extracts of cells labelled with [14C]-leucine for 15 min at 9 h after infection, together with the pattern of polypeptides in cells labelled in similar fashion and subsequently chased for 9 h in medium lacking isotope. The polypeptide patterns of uninfected cells and of purified virus are also shown. Cell protein synthesis is largely inhibited in the infected cells, allowing the complex changes occurring during the chase period to be clearly seen. The polypeptides in the infected cells are designated in Fig. 4 following a scheme based on that of Katz & Moss (1970b). Three criteria were used in identification: the relative positions of bands in the gel; the correspondence of bands in gels containing polypeptides from infected cells and purified virus; and the appearance and disappearance of bands during a chase period following a short pulse. Fig. 4 clearly shows that a close similarity exists between the polypeptide patterns...
of the chase sample and purified virus. The absence of cytoplasmic polypeptide NS3 from virus particles was a reproducible finding, however. Polypeptides P4a and Px diminished in amount during the chase and a concomitant increase was seen in the intensity of band 4b. Band 4a was only detected in chase samples and purified virus.

The changes which occurred in the polypeptide patterns during the chase period were further analysed in experiments which examined the polypeptides contained in the supernatant fluids and pellets. The results of one such experiment are shown in Fig. 5. The progressive disappearance of polypeptides P4a and Px from both fractions during the later part of the chase is a prominent feature of these gel patterns. Another polypeptide also disappeared from both fractions with similar kinetics. This polypeptide, termed Py, migrated just beyond polypeptides 6a and 6b. All polypeptides in the supernatant fluid, with the exception of NS3, were found in pellets at some stage during the chase period. Polypeptides which disappeared from both fractions have already been mentioned; pellet polypeptides which appeared during the chase and which were not found in significant amounts in the supernatant fluid included 4a and 9. The precursor–product relationship of polypeptides P4a and 4a has been demonstrated previously by Katz & Moss (1970a); the results described
here suggest that a similar relationship exists between polypeptides Py and 9, as the kinetics of their appearance and disappearance are well co-ordinated in time. Polypeptides 1a, 1b and 4b increased in the pellet fraction during the chase and a concomitant decrease in the amounts of polypeptides 1a and 1b was seen in supernatant fluids. However, during the chase no change occurred in the amount of polypeptide 4b present in the supernatant fluid. The greatly increased amounts of label in this region of the gel in the 1 h, 3 h and 9 h chase samples cannot therefore be entirely explained by movement of this polypeptide from a non-sedimentable to a sedimentable state, as previously suggested (Katz & Moss, 1970b). It is possible, however, that this polypeptide is a cleavage product of polypeptide Px, as the kinetics of appearance of 4b and disappearance of Px are well co-ordinated in time. This implies that band 4b consists of two different polypeptides with closely similar mol. wts., one remaining largely in the supernatant fluid during the chase, whereas the other, which is a major structural protein formed by the cleavage of a precursor molecule, is in particulate form at all times subsequent to its formation. Further analysis of pellets using a discontinuous buffer system, which is superior in resolution to the SDS-phosphate buffer system (Laemmli, 1970), failed to resolve two bands in this region of the gel (Fig. 6), although an increased number of bands were seen in the regions of the gel containing polypeptides in groups 5 and 6.
Release of polypeptides from sedimentable structures by treatment with NP 40

The pellets from the pulse-chase experiment described above were treated with NP 40. The resultant material was separated by centrifuging into sedimentable and non-sedimentable polypeptide fractions and these were analysed by polyacrylamide gel electrophoresis. Many polypeptides were solubilized from the pellets by NP 40 treatment (Fig. 7). These polypeptides could be grouped according to their behaviour during the chase. Polypeptides P 4a, Px and 6a, for example, were soluble in NP 40 to a considerable degree at the end of the pulse but became largely insoluble after a 15 min chase, whereas polypeptide 4a was not solubilized at any time. Polypeptide 5a remained largely insoluble throughout the experiment. Labelling of band 4b increased in amount in the supernatant fluids of the 15 min, 30 min and 1 h samples, thereafter declining, a sequence of changes similar to that found with antigen A in gel precipitin studies. In the pellets, a much more marked increase occurred from 15 min to 3 h. Significant amounts of only one polypeptide were solubilized from the 9 h chase sample. (A diffuse band difficult to reproduce photographically and not seen in the figure.) This was polypeptide 6b, previously shown by Holowczak & Joklik (1967) to be solubilized from virus particles by NP 40 treatment. The pattern of NP 40-
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Fig. 8. Autoradiograph showing the effect of rifampicin on the distribution of sedimentable polypeptides. Cells were labelled with [14C]-leucine for 15 min starting 8 h after infection. (a) No rifampicin, end of pulse; (b) no rifampicin, 15 min chase; (c) no rifampicin, 30 min chase; (d) rifampicin present throughout infection and labelling, end of pulse; (e) labelling as (d), 30 min chase; (f) labelling as (d), 15 min chase in medium without rifampicin.

Insoluble polypeptides in this sample correspond closely to that seen on electrophoresis of purified virus particles.

Effect of rifampicin on entry of polypeptides into sedimentable structures

Virus polypeptides are synthesized in the presence of rifampicin (Moss, Rosenblum & Grimley, 1971; T. H. Pennington, unpublished work), but the entry of polypeptide 4b into sedimentable structures and the cleavage of polypeptides P4a and Px are blocked (Katz & Moss, 1970a, b). This result was confirmed together with the finding by these workers that removal of the drug is followed by a rapid appearance of polypeptide 4b in particulate form (Fig. 8). Again, this behaviour resembles that of antigen A in gel precipitin experiments. Rifampicin does not prevent the entry of polypeptides P4a, Px, 5b, 6a and 6b into sedimentable structures, although these polypeptides remained largely NP40 soluble after prolonged chase in the presence of the drug (result not shown).

Identification of antigens A and B

Comparison of the polyacrylamide and gel precipitin tests described above led to the tentative identification of antigens A and B as supernatant fluid polypeptides 4b and NS3,
Table 1. Comparison of vaccinia-induced antigens and polypeptides

<table>
<thead>
<tr>
<th>Antigen or polypeptide</th>
<th>Found at some time in pellet</th>
<th>Alters in amount in supernatant fluid during chase period</th>
<th>Appearance in sedimentable form prevented by rifampicin</th>
<th>Becomes sedimentable 15 min after removal of rifampicin</th>
<th>Sedimentable form solubilized by NP40</th>
<th>NP40 soluble form increased in amount in pellet during chase period from 15 min to 1 h</th>
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<tbody>
<tr>
<td>Antigen A</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Polypeptide 4b</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Antigen B</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Polypeptide NS3</td>
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<tr>
<td>Polypeptide 4a</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<tr>
<td>Other antigens and polypeptides</td>
<td>+</td>
<td>+</td>
<td>-</td>
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* +, Possesses property indicated; -, does not possess property indicated.

Fig. 9. Autoradiograph showing polypeptides present in precipitin bands A and B. Immunodiffusion slides were washed as described in Methods and the precipitin bands were cut out using a fragment of razor blade. After boiling in 2% SDS and 1% mercaptoethanol the samples were electrophoresed in 7.5% polyacrylamide gels. (a) Bands A and B, (b) band B, (c) band A.
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Fig. 10. Co-electrophoresis of sedimentable polypeptides from rifampicin-treated cells with precipitin bands A and B. (a) Polypeptides from rifampicin-treated cells, (b) with band A, (c) with band B', (d) with bands A and B.

respectively. The data leading to these conclusions are summarized in Table 1. Direct confirmation of this identification was obtained by polyacrylamide gel electrophoresis of polypeptides solubilized from radioactive precipitin lines. Most of the radioactivity from lines A and B migrated in the gel as a single band, the line A polypeptide being of lower mol. wt. than the line B polypeptide (Fig. 9). The faint bands visible in the gels are probably contaminant polypeptides. Co-electrophoresis of precipitin bands with 18,000 g pellets prepared from cytoplasmic extracts of rifampicin-treated infected cells, which do not contain polypeptides 4b and NS3, allowed definitive identification of the two bands (Fig. 10).

DISCUSSION

Two major vaccinia induced soluble antigens have been characterized by a combined immunological and gel electrophoretic approach. Antigen B was identified as polypeptide NS3. Although synthesized in large amounts late in infection, this polypeptide appears to be non-structural. Immunodiffusion and gel electrophoresis studies showed that it remained entirely non-sedimentable throughout a long chase period and it was not detected in virus particles.

The other major precipitin band, A, contained one of the two polypeptides which migrate
in gel electrophoresis as band 4b of Katz & Moss (1970b). Both immunological and gel electrophoresis studies indicated that this polypeptide remained largely in non-sedimentable form during a long chase period and it was concluded that virus particle polypeptide 4b was probably derived by cleavage from polypeptide Px. Although remaining largely in the supernatant, some antigen A rapidly appeared in detergent-soluble form in pellets during chase experiments. After a long chase period, the amount of antigen solubilized by detergent declined to zero. Studies on the effects of NP40 on virus particles (manuscript in preparation) show that antigens A and B cannot be detected in the antigens solubilized by this detergent.

The function and ultimate fate of the portion of antigen A which can be chased into the pellet is unclear, and the possibility cannot be excluded that this antigen is a minor structural component of the virus particle and is detergent-soluble only during an intermediate stage in virus assembly. The experiments with rifampicin suggest that antigen A may be associated with immature virus particles, as the drug blocks both the formation of these particles (Moss et al. 1969; Pennington et al. 1970) and the entry of the antigen into the pellet. We are currently investigating the constitution of such pellets by electron microscopy. The rapid formation of immature virus particles following the removal of rifampicin (Grimley et al. 1970; Pennington & Follett, 1971) is paralleled by the rapid appearance of antigen A in sedimentable form following removal of the drug furnishes further supporting evidence. Critical examination of this hypothesis requires the isolation and purification of immature particles, which has not yet been achieved. The finding that antigen B is non-structural and that antigen A is not a major structural component of the virus particle recalls the observations of Westwood et al. (1965). These workers found that the strongest precipitin lines produced by vaccinia soluble antigens did not correspond to any component in a preparation of antigens solubilized from virus particles by trypsinization.

The absence from gel precipitin patterns of major precipitin lines corresponding to the large amounts of polypeptides P 4a and Px seen in gel electrophoresis patterns of the same samples may be due to poor antigenicity, poor diffusion in agar, or may be a particular characteristic of the antiserum.

The results of the polyacrylamide gel electrophoresis studies reported here confirm and extend those of Katz & Moss (1970a, b). The demonstration in this study of a possible precursor–product relationship between polypeptide Px and pellet polypeptide 4b has already been discussed. Virus particle polypeptides 4a and 4b are the two major structural components of the virus core (Katz & Moss, 1970a) and it is of interest to note that they both appear to be formed by the cleavage of a larger precursor molecule. A probable precursor–product relationship between polypeptide Py and 9 was also demonstrated.

The changes which occurred during a chase period in the distribution and in the NP40 solubility of virus particle precursor polypeptides P 4a and Px indicate that after their synthesis these polypeptides are in large part rapidly incorporated into sedimentable structures in an NP40-soluble form. Prior to their cleavage, however, these polypeptides become largely NP40-insoluble. These changes cannot at present be correlated with the morphological changes which occur during virus maturation, and require the isolation of structural intermediates in virus assembly for further elucidation. This work is now in progress.

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