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Vaccinia Virus Structural Polypeptide Derived from a High-Molecular-Weight Precursor: Formation and Integration into Virus Particles

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Polypeptide 4a, a major vaccinia structural polypeptide which was previously shown to form from a high-molecular-weight precursor is made after the period of viral deoxyribonucleic acid (DNA) synthesis. Pulse-chase experiments demonstrated that a period of 1 to 2 hr is required for a 50% conversion of precursor to product. The rates of incorporation of polypeptides into virus particles were examined. The kinetics of incorporation of labeled 4a and other major structural polypeptides into virus particles were similar, despite the additional time required for the formation of 4a from its precursor. Furthermore, 4a was present exclusively in a particulate form at all times examined. Both observations suggested that cleavage of the precursor occurs after, or immediately prior to, association with developing virus particles. Polypeptide P4a was previously identified as the probable precursor of 4a and is not ordinarily found in detectable amounts in virus particles. Under conditions in which breakdown of P4a was inhibited by adding rifampin or amino acid analogues after the period of viral DNA synthesis, isolated virus particles contained significant amounts of this polypeptide. Further analysis showed that P4a was localized within the virus core, which is also the site of 4a. Synchronization of virus assembly after the removal of rifampin was shown to be useful for studying the integration of polypeptides into a particulate fraction of the cytoplasm.

A major vaccinia virus structural polypeptide was shown by pulse-chase experiments to form from a higher-molecular-weight precursor (12). The structural polypeptide had a relatively low tryptophan content and was localized within the core of vaccinia virus particles. A rapidly labeled, tryptophan-deficient polypeptide present in cytoplasmic extracts of infected cells diminished in quantity during the chase period, and was thought to be the precursor. Since estimations, by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, of the molecular weight of the structural polypeptide and its presumed precursor were 76,000 and 125,000, respectively, a cleavage mechanism was suggested. Cleavage appeared to be a late step associated with virus maturation, as it was completely prevented by rifampin (12), the action of which is manifested at a specific stage in vaccinia virus assembly (5, 17, 19). The incorporation of structural polypeptides into vaccinia virus particles under a variety of conditions has now been examined, and particles containing the high-molecular-weight precursor have been isolated.

MATERIALS AND METHODS

Cells and virus. Suspension cultures of HeLa S-3 cells were infected with 30 plaque-forming units of vaccinia virus, strain WR, per cell (18). Virus titers were determined by plaque assay on chick embryo fibroblast monolayers with the use of an agar overlay.

Radioactive labeling. Radioactively labeled amino acids were obtained from Schwarz BioResearch, Orangeburg, N.Y. Labeling with ^{14}C-L-phenylalanine (455 mCi/mmole) was carried out in Eagle's medium (2) containing 0.01 mM phenylalanine. Labeling with ^{14}C-DL-tryptophan (34 mCi/mmole) and ^{14}C-L-leucine (316 mCi/mmole) was carried out in medium containing one-tenth the concentration of amino acids in Eagle's medium. Dialyzed horse serum was used.

^{3}H-thymidine (90 mCi/mg) was obtained from New England Nuclear Corp., Boston, Mass.

Polyacrylamide gel electrophoresis. Cytoplasmic extracts were prepared from infected cells by Dounce homogenization and then were reduced and dissociated with 2% SDS and 1% mercaptoethanol for 1 min at 100 C (15). Electrophoresis of 50- to 100-μliter samples was carried out in 7.5% polyacrylamide gels (10 cm long, 0.6 cm in diameter) containing 0.1 M sodium phosphate (*p*H 7.1) and 0.1% SDS at 3 ma

per gel for 15 hr. The gels were washed with trichloroacetic acid (10%) and acetic acid (7.5%), sliced longitudinally, dried, and placed in contact with X-ray film (Kodak, no screen; 4). Tracings of the radioautographs were made with a Joyce-Loebl microdensitometer.

Preparation of purified virus and cores. Virus was purified from cells which were disrupted by Dounce homogenization. The cytoplasmic fraction obtained after low-speed centrifugation was subjected to sonic vibrations of 20 kc for 1 min and then was sedimented through 36% (w/v) sucrose in 10 mM tris(hydroxymethyl)aminomethane (Tris), *p*H 9.0, in an SW 50L rotor at 18,000 rev/min (25,000 × *g*) for 1 hr. The pellet was resuspended and layered on a 25 to 40% sucrose gradient. After centrifugation at 13,000 rev/min (14,000 × *g*) for 35 min, the virus band was collected, diluted with 10 mM Tris (*p*H 9.0), and centrifuged at 18,000 rev/min (25,000 × *g*) for 1 hr. In most experiments, the latter two centrifugations were repeated.

Cores were prepared by a modification of a procedure described by Easterbrook (3). Radioactively labeled purified virus particles were incubated for 30 min at 37 C in a solution containing 50 mM Tris (*p*H 8.6), 0.5% NP-40 detergent (Shell, London), and 50 mM dithiothreitol. The mixture was then sonically treated and layered onto 3.5 ml of 36% (w/v) sucrose in 10 mM Tris (*p*H 8.6) containing 2 mM dithiothreitol. Samples from the top of the tube and from the core-containing pellets were taken after centrifugation in an SW 50L rotor at 18,000 rev/min (25,000 × *g*) for 80 min.

DL-Fluorophenylalanine and DL-ethionine were obtained from Sigma Chemical Co., St. Louis, Mo. L-Canavanine sulfate and L-azetidine-2-carboxylic acid were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and Calbiochem, Los Angeles, Calif., respectively. Rifampin was a gift from Dow Chemical Co., through the courtesy of R. B. Nolan.

RESULTS

Synthesis of structural and nonstructural polypeptides. The time of synthesis of vaccinia virus structural polypeptides can be determined by pulse-labeling cells at various times after infection and isolating virus at the end of the growth cycle (6, 21). Previous experiments, which established that structural polypeptide 4a is synthesized from a precursor, were carried out at relatively late times in the vaccinia virus growth cycle (12). In the following experiment, HeLa cells were pulse-labeled with ^{14}C-phenylalanine for 10 min at various times after infection. At each time, the labeled polypeptides, present in the cytoplasm immediately after the pulse and after a long chase with unlabeled phenylalanine, were compared with structural polypeptides extracted from virus particles purified at the latter time. Radioautographs of SDS-polyacrylamide gels are shown in Fig. 1. First, the polypeptides incorporated into

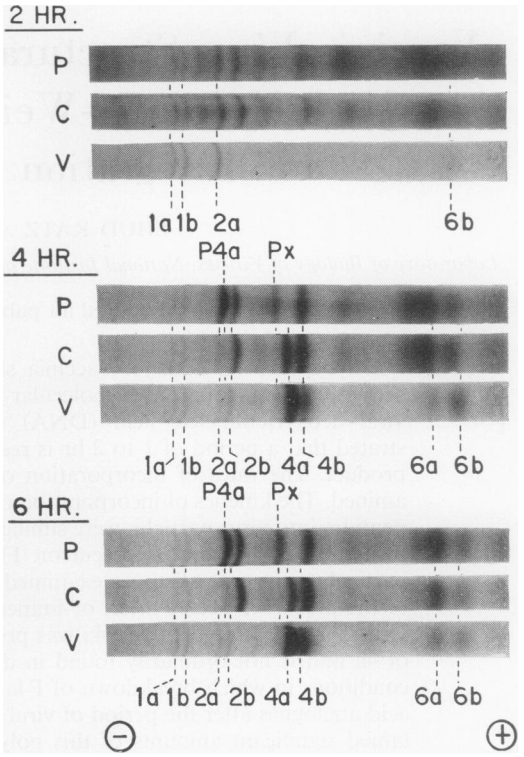

FIG 1. *Synthesis of structural and nonstructural polypeptides. At 2, 4, and 6 hr after vaccinia virus infection, portions of cells were labeled with 5 μCi of ^{14}C-phenylalanine per ml for 10 min. The cells were then washed and resuspended in medium containing 100 times the original concentration of unlabeled phenylalanine. Samples were removed at the end of the pulse and at 23 hr after infection. The polypeptides present in the total cytoplasm at each time, as well as in virus from the 23-hr sample purified by repeated sucrose gradient centrifugations, were solubilized with SDS and mercaptoethanol and separated by polyacrylamide gel electrophoresis. Raadioactive bands were demonstrated by radioautography. Polypeptides with molecular weights of less than 30,000 are not present in the portion of the radioautographs shown. P, total cytoplasmic polypeptides labeled during the 10-min pulse; C, total cytoplasmic polypeptides at the end of the chase; V, purified virus.*

virus particles will be considered. Because of the wide variations in the molecular weights of the vaccinia virus structural polypeptides, it is difficult to resolve all of them as sharp bands on a single gel. Only polypeptides with molecular weights of 30,000 and above are shown in these radioautographs. Those polypeptides which will be specifically referred to in the text are indicated by dotted lines in the figures. The polypeptide of central interest in this study is designated 4a. This polypeptide, as well as all other structural poly-

peptides resolved, appeared to be continuously made from 4 hr on, although not at constant rates (Fig. 1, V).

A more restricted group of structural polypeptides were made at 2 hr after infection (Fig. 1, V). Polypeptides 1a, 1b, 2a, and 6b were the most prominent and correspond to VSP1, VSP2, and VSP6 previously shown to be early proteins by Holowczak and Joklik (6). Additional bands could be seen after prolonged exposure of the radioautographs. These early structural polypeptides continued to be made throughout infection, differing in this respect from other early vaccinia proteins (10, 13, 14, 18, 21, 24). The same structural polypeptides made at 2 hr were also made in the presence of 5-fluorodeoxyuridine (5-FUDR) and were incorporated into virus particles after the addition of thymidine (Fig. 2). The inhibition of vaccinia virus deoxyribonucleic acid (DNA) synthesis by 5-FUDR and the reversal by thymidine have been previously described (20). It is evident that polypeptide 4a is not made prior to viral DNA synthesis (9, 20).

It is possible to identify the labeled structural polypeptides in the cytoplasm, as well as virus-directed nonstructural polypeptides, because host protein synthesis is inhibited after vaccinia infection (6, 16, 18, 21, 23). The labeled polypeptides present in the total cytoplasm immediately after the pulse and at the end of the chase were separated by SDS-polyacrylamide gel electrophoresis (Fig. 1, P and C). Very obvious changes occurred in the cytoplasmic polypeptides during the chase period (Fig. 1). The most prominent were the

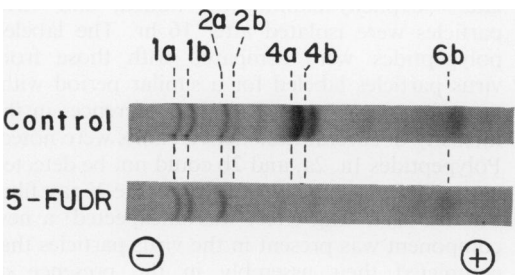

FIG. 2. *Synthesis of structural polypeptides in the presence of 5-FUDR. HeLa cells were treated with 10^{-6} M 5-FUDR 10 min prior to infection. ^{14}C-phenylalanine (0.5 μCi/ml) was added 30 min after infection, and 4.5 hr later the cells were washed and resuspended in medium containing thymidine (10^{-6} M) and ^{12}C-phenylalanine. At 22 hr after infection, the cells were harvested, and virus was purified. Virus was also purified from a culture not treated with 5-FUDR (control). The polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis. Radioautographs are shown.*

disappearance of polypeptides P4a and Px and the appearance of 4a. Polypeptide P4a is thought to be the precursor of 4a, because both have low tryptophan contents as determined by labeling with a variety of radioactive amino acids (12). When pulse-labeled cytoplasmic polypeptides containing P4a were mixed with purified virus prior to electrophoresis, the position of P4a on the radioautograph was between the structural polypeptides 2a and 2b. If P4a is contained in normal virus particles, the amount is below our limits of detection.

The rates at which P4a disappears and 4a appears after a 10-min pulse with ^{14}C-phenylalanine are shown in the following experiment. Quantitation was performed by determining the density of the X-ray film radioautographs with a double-beam recording microdensitometer. After a short lag, the decrease in peak density of P4a and increase in peak density of 4a occurred in an approximately linear fashion for about 3 hr (Fig. 3). The initial density of 4a is actually a background value caused by two adjacent polypeptides. The close spacing of the radioactively labeled polypeptide bands prevented accurate determination of the areas under each peak. Since the width of each band was different, the peak heights could not be used to compare the total amounts of the different polypeptides.

Integration of structural polypeptides into virus particles. The incorporation of structural polypeptides into virus particles was determined by pulse-labeling cells with ^{14}C-phenylalanine for 10 min at 8 hr after infection. Although virus particles containing labeled polypeptides could be isolated within 30 min after addition of the radioactive amino acids, formation of these particles may have started before the pulse. The labeled polypeptides contained in virus particles isolated at various times after the pulse are shown in Fig. 4. The kinetics of incorporation of 4a, 4b, 6a, and 6b appeared similar (Fig. 5).

A portion of the cytoplasm at each time was also centrifuged at a speed (20,000 × *g* for 15 min) sufficient to sediment virus particles. In contrast to other structural polypeptides, which were found in both supernatant and pellet fractions, polypeptide 4a was found exclusively in the pellet fractions, even at the earliest times.

The similar kinetics of incorporation of labeled 4a and other major structural polypeptides into virus particles, despite the additional time required for the formation of 4a from its precursor and the presence of 4a exclusively in particulate form, suggested two alternatives. Either 4a is incorporated into developing virus particles immediately after formation or the precursor is

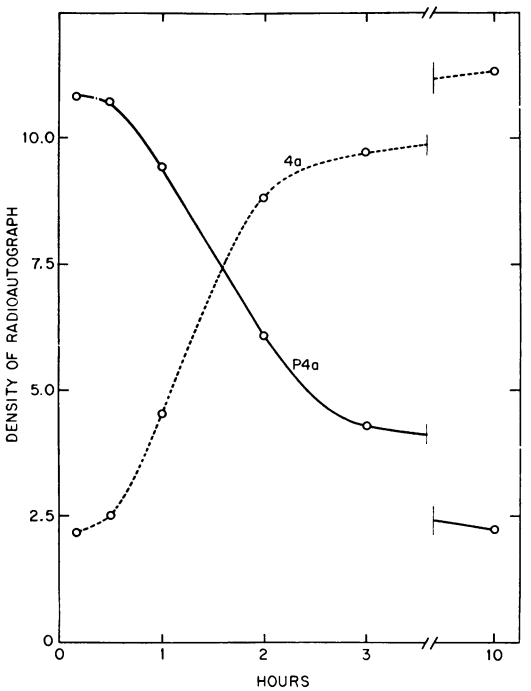

FIG. 3. *Changes in labeled P4a and 4a in the cytoplasm of infected cells after a short pulse. Infected HeLa cells were pulse-labeled with ^{14}C-phenylalanine (6 μCi/ml) for 10 min, starting at 8 hr after infection. The cells were then washed and resuspended in media containing excess unlabeled phenylalanine. At intervals, equal portions were removed, cytoplasmic extracts were prepared, and the polypeptides were separated by gel electrophoresis. The X-ray film radioautographs were analyzed by use of a Joyce-Loebl microdensitometer with a 5× scale enlargement. The heights of the peaks corresponding to P4a and 4a are expressed in centimeters. The density of a stable nonstructural polypeptide migrating just ahead of P4a was used to normalize the densities of P4a and 4a on the same gel.*

incorporated into developing virus particles where cleavage subsequently occurs.

Interruption and synchronization of virus assembly with rifampin. In the presence of rifampin, virus-directed polypeptides are synthesized but virus assembly is blocked (17). Cleavage of P4a does not occur, and polypeptide 4a cannot be detected (12). When rifampin is removed, virus assembly begins synchronously within 2 min, as judged by electron microscopic observations (5), but formation of 4a is delayed 30 to 60 min (Fig. 6). This is approximately the time required for the detection of mature virus particles.

The polypeptide composition of cytoplasmic fractions was examined in an effort to detect early changes after the removal of rifampin. When rifampin was removed, virtually all of the increasing deoxyribonuclease-resistant viral DNA was localized in the 20,000 × *g* particulate fraction, suggesting that it contains developing viral forms (Table 1). Analysis of the polypeptides revealed that the structural polypeptide 4b was not in a particulate form in the presence of rifampin (Fig. 7). After removal of the drug, this polypeptide appeared in the particulate fraction very rapidly, a significant amount of it accumulating within 10 min. This even preceded the formation of 4a (Fig. 8), and is presumably an earlier step in maturation. Deoxyribonuclease treatment of the particulate fraction, as in Table 1, did not relase a significant amount of labeled polypeptides. Both 4a and 4b are localized in the core of mature virus particles (12).

Formation of virus particles, after the addition of rifampin, containing polypeptide P4a. In the previous experiments, rifampin was added at the start of the infection. When rifampin is added at 6 hr, formation of new virus particles is interrupted but immature virus particles already present at that time appear to continue their development (5). We considered it possible that virus particles which were already in the process of maturation could be specifically labeled by adding radioactive amino acids after the addition of rifampin. The relative amounts of labeled structural polypeptides in these particles would then be altered, so that more radioactivity would be present in polypeptides integrated at late stages of assembly. In the following experiment, rifampin was added 6 hr after infection; 1 hr later ^{14}C-phenylalanine was added, and virus particles were isolated after 16 hr. The labeled polypeptides were compared with those from virus particles labeled for a similar period without rifampin (Fig. 9). Relative differences in the intensity of several radioactive bands were noted. Polypeptides 1a, 2a, and 2b could not be detected even after prolonged exposure of the X-ray film. The most striking effect was unexpected: a new component was present in the virus particles that completed their assembly in the presence of rifampin. The position of this polypeptide in the gel corresponded precisely with that of P4a. In stained polyacrylamide gels which also contained polypeptides of normal carrier virus, the new component was seen as a distinct band between 2a and 2b. The identification of both P4a and 4a was further established by their low incorporation of tryptophan (Fig. 9). An increased amount of another polypeptide with low tryptophan content, just ahead of P4a, was also noted.

Polypeptide 4a is found in the core of vaccinia

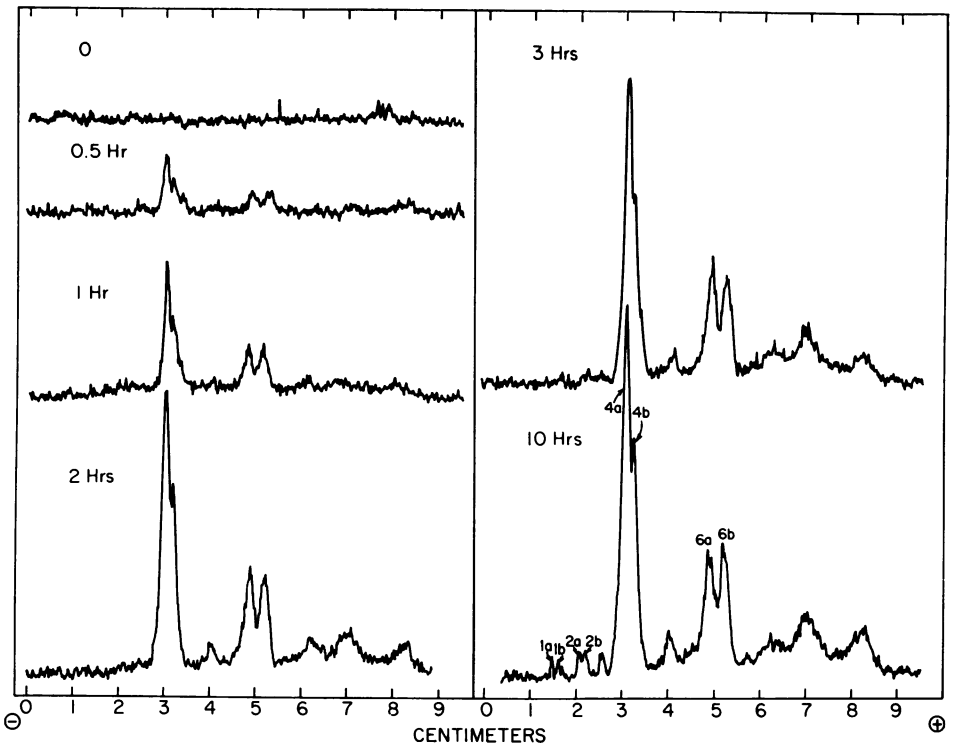

FIG. 4. *Incorporation of polypeptides into virus particles. Carrier unlabeled virus was added to the samples of cells taken at intervals during the chase period described in Fig. 3. The labeled virus was purified and the polypeptides were analyzed by gel electrophoresis. Densitometer tracings of the radioautographs are shown.*

virus particles (12), and similar location of P4a in these particles was also demonstrated (Fig. 10).

Effect of amino acid analogues on the formation of polypeptide 4a. Incorporation of amino acid analogues into poliovirus precursor polypeptides prevents their subsequent cleavage (7, 8). Fluorophenylalanine, canavanine, ethionine, and azetidine-2-carboxylic acid, which are analogues of phenylalanine, arginine, methionine, and proline, respectively, were added separately and together to vaccinia virus-infected HeLa cells to determine their effect on the formation of 4a. The analogues were present between 7 and 7.5 hr of infection. ^{14}C-leucine was present during the last 20 min of this period. The radioactive amino acid pulse and the period of analogue incorporation were terminated by washing the cells and resuspending them in medium containing excess quantities of unlabeled normal amino acids. A full yield of infectious virus was obtained because the analogues were present for only a short time. Polypeptides made before or after the addition of analogues were not labeled under these conditions. Radioautographs showing cytoplasmic viral polypeptides immediately after the pulse and after a 4-hr chase are presented in Fig. 11. Breakdown of P4a and formation of 4a were prevented when all four analogues were used simultaneously and were partially inhibited by fluorophenylalanine, canavanine, and azetidine-2-carboxylic acid alone. A surprising result was found when the labeled polypeptides incorporated into virus particles were examined. Although a polypeptide corresponding to 4b was synthesized when the cells were treated with either fluorophenylalanine or canavanine, it was not incorporated into virus particles (Fig. 11). Under these conditions, other polypeptides, including 4a, which was further identified by its low tryptophan labeling, were incorporated. We presume that unlabeled polypeptide 4b made before or after the addition of analogues was incorporated preferentially, perhaps because of an altered conformation of the analogue-containing polypeptide. Similarly, use of canavanine alone prevented the incorporation of labeled 6a into virus particles (Fig. 11).

Formation in the presence of fluorophenylalanine, of virus particles containing polypeptide P4a. We also hoped to use fluorophenylalanine

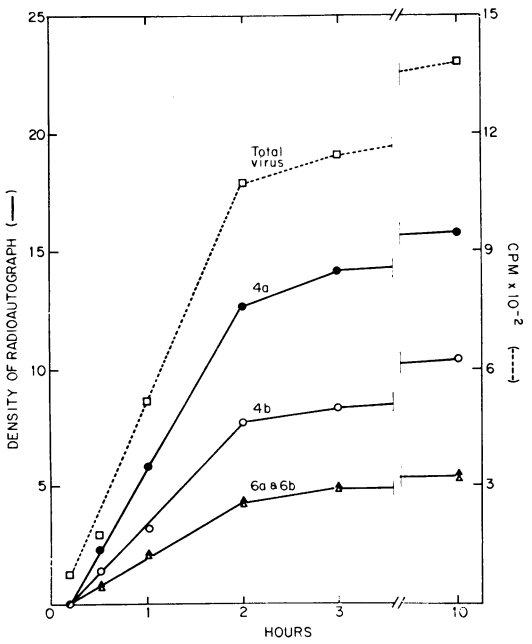

FIG. 5. *Relative rates of incorporation of structural polypeptides into virus particles. The X-ray film radioautographs used for Fig. 4 were traced by use of a microdensitometer with a 5× enlargement to better separate the peaks. The heights of the peaks are expressed in centimeters. Total incorporation of ^{14}C-phenylalanine into purified virus particles was determined by scintillation counting.*

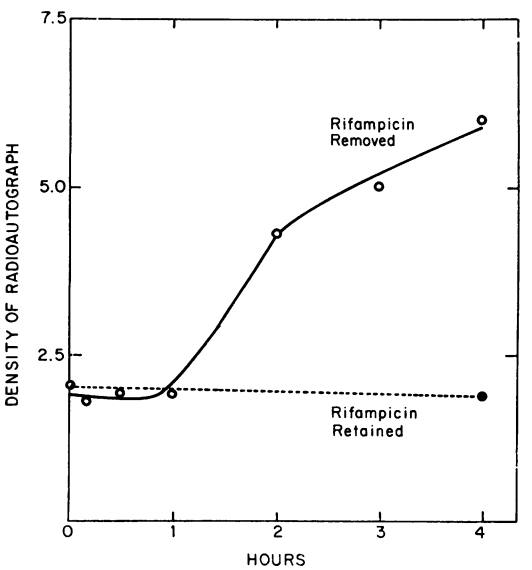

FIG. 6. *Formation of 4a after the removal of rifampin. HeLa cells treated with rifampin (100 μg/ml) starting*

TABLE 1. *Localization of deoxyribonuclease-resistant viral DNA after the removal of rifampin*[a]

Time after removal of rifampin (min)	DNA in pellet (%)	Deoxyribonuclease-resistant (%)	
		Pellet	Supernatant
0	6.7	7.1	7.1
15	7.3	15.3	8.9
60	7.8	35.5	6.9
120	12.2	50.1	6.1
180	16.2	78.8	6.6
Control[b]	7.7	17.7	5.9

[a] HeLa cells were treated with rifampin (100 μg/ml) 10 min prior to infection. At 2 hr after infection, 0.15 μCi of ^{3}H-thymidine per ml was added; 2 hr later, the ^{3}H-thymidine was removed by washing and resuspending the cells in medium containing 10^{-6} M unlabeled thymidine. At 7.5 hr after infection, rifampin was washed from one portion of cells and retained in another. The incubation was continued, and at intervals cells were washed, homogenized in 1 mM phosphate buffer (*p*H 7.0), and centrifuged at 20,000 × *g* for 15 min. The amounts of trichloroacetic acid-precipitable radioactive material in the cytoplasmic extract and in the pellet were determined. Portions of the supernatant and the suspended pellet were treated with 100 μg of deoxyribonuclease I (Worthington Biochemical Corp., Freehold, N.J.) per ml in the presence of 10 mM $MgCl_2$ for 30 min at 37 C. The percentage of trichloroacetic acid-precipitable material resistant to this treatment was calculated.

[b] Rifampin was present for 180 min.

in a manner analogous to the use of rifampin, to form virus particles retaining uncleaved P4a. A previous study by Salzman, Shatkin, and Sebring (22) of the effects of this analogue on the formation of infectious vaccinia virus was useful in planning the following experiment. At 4 hr after infection, a time when most of the viral DNA has been made and late protein synthesis has recently begun, the cells were resuspended in

10 min prior to infection were incubated with 0.25 μCi of ^{14}C-phenylalanine per ml from 3.5 to 7.5 hr. The infected cells were then washed and resuspended in media containing excess unlabeled phenylalanine and without rifampin. At intervals, portions were homogenized, the cytoplasmic extracts were treated with SDS and mercaptoethanol, and the polypeptides were separated by gel electrophoresis. A portion of cells from which rifampin was not removed served as control. The height of the peak (centimeters) corresponding to 4a was measured from densitometer tracings of the radioautographs.

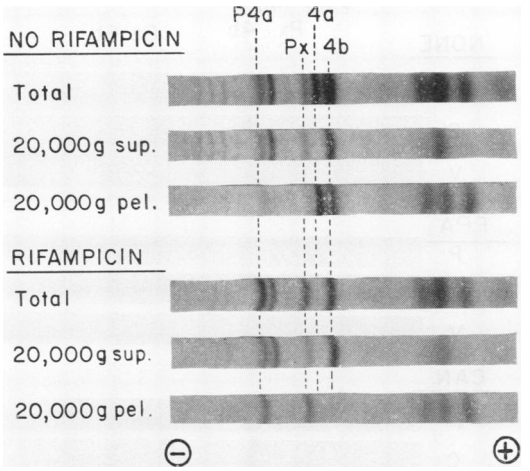

FIG 7. *Polypeptides associated with a 20,000 × g particle fraction. HeLa cells were treated with rifampin (100 μg/ml) starting 10 min prior to infection. A portion of untreated infected cells served as control. At 4.5 hr after infection, 0.5 μCi of ^{14}C-phenylalanine per ml was added. At 8 hr after infection, the cells were homogenized and cytoplasmic extracts were prepared. Large aggregates were disrupted with sonic vibrations at 20 kc for a total of 1 min, and the extract was centrifuged for 15 min at 20,000 × g. The supernatant was collected, and the pellet was resuspended in an equivalent volume. The cytoplasmic extract (total) and the supernatant and pellet fractions were treated with SDS and mercaptoethanol, and the polypeptides were separated by gel electrophoresis. Radioautographs are shown.*

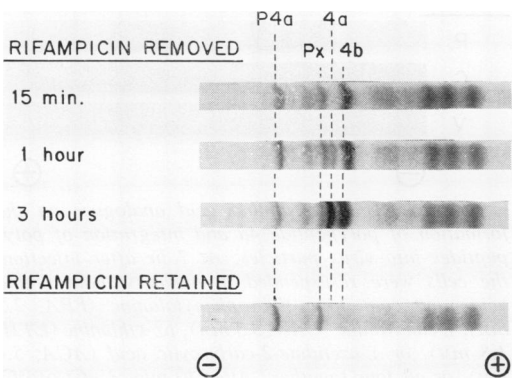

FIG. 8. *Polypeptides integrated into the 20,000 × g particle fraction after removal of rifampin. Cells were treated with rifampin (100 μg/ml) 10 min prior to infection. At 4 hr after infection, 0.25 μCi of ^{14}C-phenylalanine per ml was added; 3 hr later, the cells were washed and resuspended in medium containing excess unlabeled phenylalanine with or without rifampin. At intervals, portions were taken, and the cytoplasmic extracts were separated into particle and supernatant fractions, as in Fig. 7, for gel electrophoresis.*

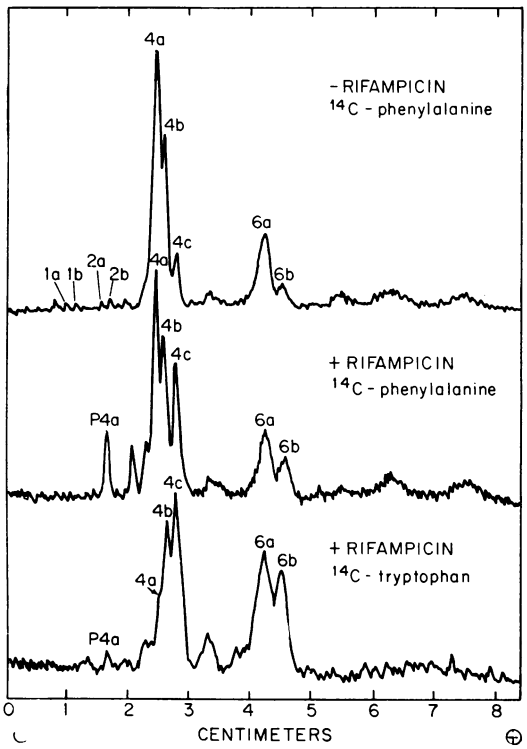

FIG. 9. *Integration of P4a into virus particles in the presence of rifampin. Infected cells were treated with rifampin (100 μg/ml) starting at 6 hr after infection. ^{14}C-phenylalanine (1.5 μCi/ml) or ^{14}C-tryptophan (1 μCi/ml) was added 1 hr later, and the cells were harvested after an additional 16 hr. An infected culture also labeled with ^{14}C-phenylalanine but not treated with rifampin served as a control. Virus was purified and the polypeptides were separated by polyacrylamide gel electrophoresis. Densitometer tracing of the radioautographs are shown. Several polypeptides are indicated by arrows. The small amount of tryptophan labeled 4a appears as a small shoulder preceding 4b.*

medium containing a mixture of fluorophenylalanine (0.25 mM) and phenylalanine (0.01 mM). Under these conditions, formation of infectious virus continued for only a short while (Fig. 12), and formation of ^{3}H-thymidine-labeled virus particles, (^{3}H-thymidine was present from 30 min to 4 hr after infection) was reduced by 92.6%. We considered that some particles formed might be defective and perhaps contain uncleaved P4a. In the next experiment, ^{14}C-leucine was added 30 min after the analogue, and virus particles were isolated from the infected cells 17 hr later. The polypeptides were separated by SDS-polyacrylamide gel electrophoresis. The results indicated that polypeptide P4a was present in purified virus particles formed under these conditions. Photo-

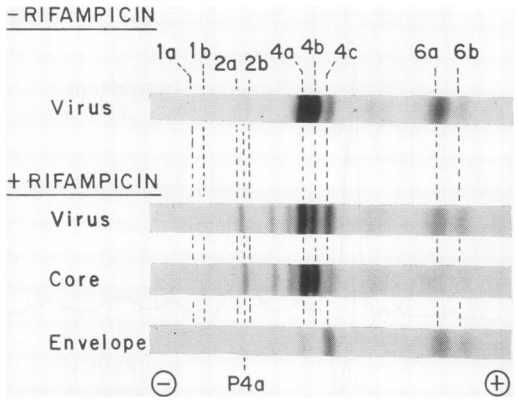

FIG. 10. *Localization of P4a within the virus core. Virus labeled with ^{14}C-phenylalanine after the addition of rifampin, as described in Fig. 9, was treated with NP-40 and mercaptoethanol. The unreleased (core) and the released (envelope) polypeptides were separated by centrifugation and analyzed by gel electrophoresis. Radioautographs of the gels are shown.*

graphs of Coomassie Blue-stained gels and radioautographs are shown in Fig. 13. The polypeptide located between 2a and 2b is in the exact position of P4a. A double label experiment with ^{14}C-phenylalanine and ^{3}H-tryptophan indicated that this polypeptide was deficient in tryptophan and was associated with the virus core. Experiments with ^{14}C-fluorophenylalanine showed that this analogue was incorporated into P4a as well as other structural polypeptides.

DISCUSSION

The formation of structural polypeptide 4a from a high-molecular-weight precursor was established by pulse-chase experiments (12). Similar experiments showed that two nonstructural polypeptides P4a and Px were converted to another form during the chase period. It seems likely that P4a is converted to 4a, since both show a relative deficiency in the same amino acid, tryptophan (12). Estimations of the molecular weights by SDS-gel electrophoresis indicated that a 40% reduction in the size of P4a is needed to form 4a. Purification and peptide analysis will be required to determine the exact relationship of P4a and 4a.

Polypeptides P4a and 4a are made subsequent to the onset of viral DNA synthesis. At 8 hr after infection, a 50% conversion of newly synthesized precursor to 4a takes between 1 and 2 hr. Under these conditions, a lag period of less than 30 min occurs before formation of 4a can be detected. Since other structural polypeptides are formed directly, a delay in the incorporation

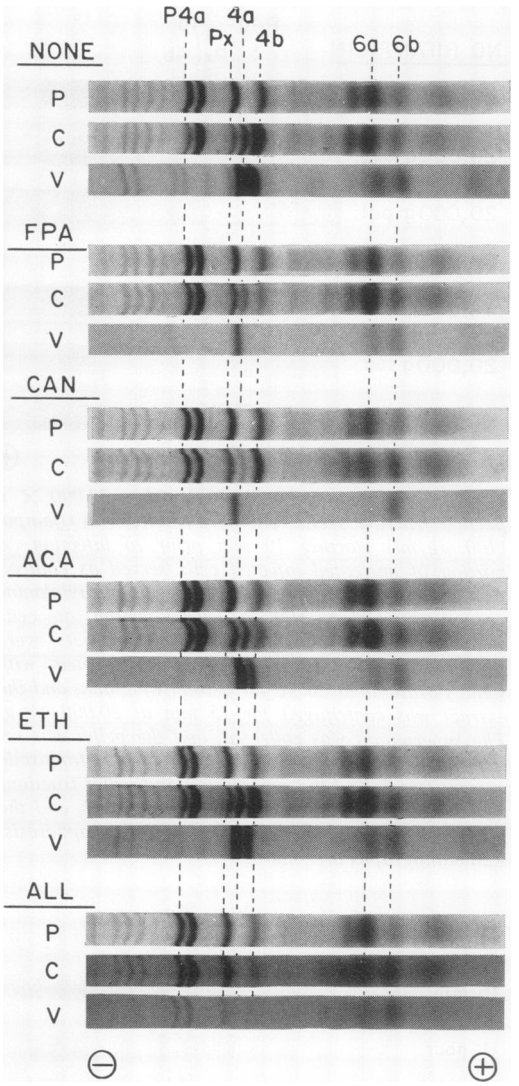

FIG. 11. *Effect of amino acid analogues on the formation of polypeptide 4a and integration of polypeptides into virus particles. At 7 hr after infection, the cells were resuspended in media without amino acids and containing fluorophenylalanine (FPA, 2.5 mM), L-canavanine (CAN, 3.3 mM), DL-ethionine (ETH, 1.8 mM), or L-azetidine-2-carboxylic acid (ACA, 5.4 mM), or all four together. After 10 min, 3 μCi of ^{14}C-leucine per ml was added. After 20 additional min, the cells were washed and resuspended in medium containing excess normal unlabeled amino acids. At the end of the pulse and at 4 and 14 hr later, portions were removed and cytoplasmic extracts were prepared. Virus was purified at the later time. Polypeptides in the cytoplasmic extracts from samples taken at the end of the pulse and after a 4-hr chase, and polypeptides in the purified virus, were separated by gel electrophoresis. Radioautographs are shown.*

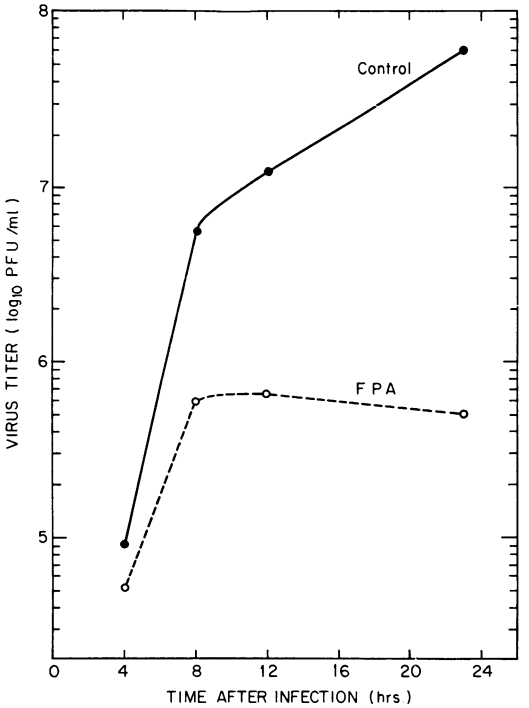

FIG. 12. *Formation of infectious vaccinia virus after addition of fluorophenylalanine. Infected cells were resuspended in medium containing fluorophenylalanine (0.25 mM) and phenylalanine (0.01 mM) at 4 hr after infection. A culture to which fluorophenylalanine was not added served as a control. Samples were removed at intervals and the infectious virus titers were determined by plaque assay.*

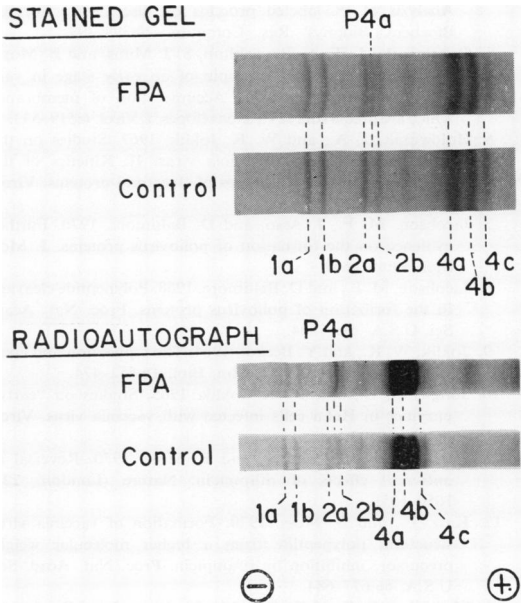

FIG. 13. *Integration of P4a into virus particles in the presence of fluorophenylalanine. Infected cells were resuspended in medium containing 0.25 mM fluorophenylalanine and 0.01 mM phenylalanine at 4.5 hr after infection; 30 min later, ^{14}C-leucine (1 μCi/ml) was added. An infected culture which was not treated with the analogue served as a control. The cells were harvested 22 hr after infection; virus was purified and the polypeptides were separated on polyacrylamide gels. Gels stained with Coomassie brilliant blue in 10% trichloroacetic acid (1) and autoradiographs are shown. Note that they are not the same scale.*

of 4a into virus particles and different kinetics of incorporation for these two classes of proteins might have been expected. In fact, the kinetics of incorporation of the majority of structural polypeptides were similar. This finding and the detection of 4a exclusively in a particulate form would be expected if the precursor were incorporated into developing particles at the same time as other structural polypeptides and was subsequently cleaved.

A requirement for particle assembly for the formation of 4a was previously shown by use of rifampicin (12). The most direct evidence for the actual integration of P4a into developing virus particles was the finding that, under conditions in which formation of 4a and assembly are disturbed, either by adding rifampin at late times in infection or by adding amino acid analogue, the virus particles isolated still contain the precursor polypeptide. P4a was localized within the virus core, which is also the location of 4a.

Synchronization of virus assembly after the removal of rifampin was very useful for studying virus development at a morphological level. Immature virus particles are formed within 10 min after drug removal. In the present study, examination of disrupted cytoplasmic fractions indicated that polypeptide 4b moved from a nonparticulate to a particulate form during this short time. This event preceded the formation of 4a and the rise in particulate ribonucleic acid polymerase activity (11). Isolation of specific intermediate viral forms may help elucidate the steps in vaccinia virus assembly.

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