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Isolation of Vaccinia Virus Mutants Capable of Replicating Independently of the Host Cell Nucleus†

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α-Amanitin-resistant vaccinia virus mutants were isolated after serial virus passages in BSC-40 cells that were carried out in the presence of inhibitory levels (6 μg/ml) of α-amanitin. One such mutant, α-27, was highly refractory (>95%) to α-amanitin-mediated inhibition and was selected for further study. In the absence of drug, the phenotypes of α-27 and wild-type vacinnia virus were indistinguishable with respect to growth kinetics, DNA synthesis, protein synthesis, and morphogenesis. Infections in the presence of α-amanitin revealed two striking differences, however. First, wild-type virus was unable to catalyze proteolytic processing of the two major capsid proteins VP62 and VP60, whereas α-27 was most efficient at this process. Second, wild-type viral morphogenesis within the infected cells was arrested by α-amanitin at an apparently analogous step to that previously described for enucleated cells. This observation was supported by the ability of α-27 virus to replicate in enucleated BSC-40 cells. Restriction enzyme analyses of α-27 versus wild-type genomes revealed that a *XhoI* cleavage site was altered in the α-27 DNA molecule, suggesting a possible location for the α-amanitin resistance locus.

Traditionally, the poxviruses, such as vaccinia virus (VV), have been considered to be the sole representatives of the DNA-containing animal viruses which were capable of replicating independently of the host cell nucleus (9). This concept was based on two lines of evidence. First, virtually all the virus-specific replicative events that could be detected, either biochemically or with the electron microscope, occurred within the cytoplasmic compartment of infected cells. Second, VV virions contain or encode many of the enzymes necessary for viral DNA and RNA biogenesis, implying nuclear independence (13). This idea was supported by the experiments of Prescott et al. (16), who demonstrated the occurrence of VV DNA replication in enucleated mouse L-cells.

The first indication that the situation was not quite so straightforward was provided by the experiments of Pennington and Follett (15) which showed that VV infections of enucleated cells did not result in the production of infectious progeny as might have been expected. More recently, the question of nuclear involvement in poxvirus replication has been reexamined in greater detail (9, 10, 19, 20). These studies showed that whether the nucleus of a cell was physically removed by cytochalasin B-mediated enucleation, functionally inactivated by UV irradiation, or selectively inhibited with α-amanitin (an inhibitor of host but not viral transcription), the results were identical upon subsequent VV infection. VV was able to enter the cells and initiate the infection, early genes were expressed, viral DNA was replicated, and late genes were expressed; however, at this point the virus encountered some sort of block and was unable to complete its morphogenetic development into mature infectious progeny virions. It was not clear whether this inhibition was due to an aberration in a late replicative step or whether it was due to an early defect which only becomes apparent at late times after infection. In either case, two theories were proposed to explain the apparent requirement for the host transcriptional apparatus during VV replication. Dales and his co-workers have suggested that the host cell RNA polymerase II is needed to transcribe a portion of the VV genome at late times during infection (20). This hypothesis is strengthened by the observations of Bolden et al. (3), who detected both VV DNA and RNA within the nuclear compartment of infected cells. Alternatively, Hruby et al. (10) hypothesized that activity of the transcriptional system of the host was needed to supply a labile factor which participated directly in the viral assembly process. Unfortunately, it was difficult to conceive experiments sufficient to distinguish between these, and other, theories as to the nature of the host cell nuclear involvement during productive poxvirus infection.

Recently, however, the utility of a new approach to studying VV genes of interest has become obvious. Several laboratories (11, 17, 23) have used selection of drug-resistant VV mutants as a procedure to target mutations into specific genes of interest. For example, selection of phosphonoacetic acid-resistant VV mutants has led to the mapping and characterization of the viral DNA polymerase gene (11, 23). In the experiments reported in this paper, we have pursued a similar avenue toward unraveling the question of nuclear involvement. Specifically, we have sought to select and isolate α-amanitin-resistant (α-A^r) VV mutants. We reasoned that such mutants might prove useful in at least two ways. First, a detailed biochemical comparison between wild-type (WT) and drug-resistant VV might provide more specific information concerning the nature of the nuclear requirement. And second, by using marker rescue techniques (5) with the dominant drug-resistant phenotype, it should be possible to locate and identify the VV gene(s) which requires the activity of the host cell nucleus.

MATERIALS AND METHODS

Cells and viruses. BSC-40 cells, a clonal derivative of BSC-1 cells selected for their ability to grow at 40°C, were grown in monolayers and maintained with Eagle minimum essential medium (Flow Laboratories) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 50 μg of gentamicin sulfate per ml. Parental VV (WR strain) was

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obtained from the American Type Culture Collection in 1977 and has been propagated by low-multiplicity passages and periodic plaque purification. Hydroxylamine (HA)- and nitrosoguanidine (NG)-mutagenized VV was prepared and kindly provided by R. Condit (4). Viral infections and plaque assay titrations were performed as previously described (9).

Mutant isolation. Confluent monolayers of BSC-40 cells (60-mm dishes) were treated with 6 μg of α-amanitin (Sigma Chemical Co.) per ml for 18 h before infection to equilibrate the effective drug dosage (10). The pretreated cells were then infected at a multiplicity of 1 with WT or mutagenized stocks of VV. After 72 h of infection in the presence of drug, the infected cells were harvested into 4 ml of sterile phosphate-buffered saline. Progeny virions were liberated by two cycles of freeze-thawing. Titers were determined for a portion of this crude stock while 0.5 ml was used to infect another set of α-amanitin-treated BSC-40 cells. This set of steps was repeated a total of seven times. Note that although the initial multiplicity was 1, subsequent passages by necessity were carried out at much lower multiplicities due to α-amanitin-mediated reduction in crude stock titer.

After seven serial passages, drug-resistant virus populations had arisen from each of the three parental stocks (WT, HA, and NG). Twenty-five individual α-A^r mutants were isolated from each mixture by the following procedure. Cell monolayers (100 mm) were infected with ca. 100 PFU of α-A^r VV. After 48 h, the monolayers were stained with 0.01% neutral red, and 25 well-isolated plaques were picked with sterile Pasteur pipettes. Fifteen (times three) of the plaque isolates were frozen at −70°C for later analysis. The remaining 10 (times three) were used to infect BSC-40 monolayers and to produce crude stocks whose titers were subsequently determined. From these 30 α-A^r mutants, the 15 having the highest titers were chosen for further analysis, whereas the other 15 were frozen. The entire plaque isolation procedure was carried out in the absence of drug selection.

Mutant characterization. (i) Virus growth. Duplicate 60-mm dishes of BSC-40 cells were pretreated with the indicated concentrations of α-amanitin for 18 h before infection. The monolayers were then infected (for 24 h at 37°C) with identical multiplicities of WT or α-A^r VV. The infected cells were harvested into 4 ml of sterile phosphate-buffered saline and subjected to two cycles of freeze-thawing. The infectious titers were determined by plaque assay of serial virus dilutions on BSC-40 monolayers in the absence of drug. Viral plaques were visualized by staining with either neutral red or crystal violet (9).

(ii) Protein synthesis. BSC-40 monolayers were infected with VV at a multiplicity of 25 and then maintained in complete medium at 37°C. At the indicated times postinfection, the medium was replaced with medium (minus methionine) containing 5 μCi of L-[^{35}S]methionine (New England Nuclear Corp.; 1,143 Ci/mmol) per ml and incubated at 37°C for 30 min. The cells were then loosened with a rubber policeman, harvested by low-speed centrifugation and suspended in 0.5 ml of ice-cold 1 mM Tris-hydrochloride (pH 9), and the hot-trichloroacetic acid-precipitable radioactivity of an appropriate sample was determined. Samples containing equivalent radioactive counts were then analyzed by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels (22) and autoradiography of the dried gels on Kodak XAR-5 film.

(iii) DNA synthesis. BSC-40 monolayers were infected with VV and incubated at 37°C. At various times postinfection, 5 μCi of [*methyl*-^{3}H]thymidine (New England Nuclear Corp.; 78.1 Ci/mmol) per ml was added to the medium for 10 min.

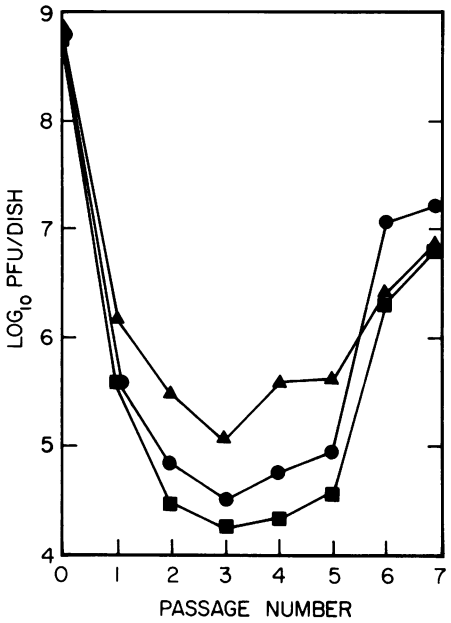

FIG. 1. Serial passage of VV in the presence of α-amanitin. Cell monolayers were pretreated with 6 μg of α-amanitin per ml for 12 h and then infected with VV (WT [▲] or mutagenized with HA [●] or NG [■]) at a multiplicity of 1. After 72 h of infection in the presence of the drug, progeny virus was harvested, titers were determined, and the progeny virus was used to reinfect α-amanitin-treated monolayers. This procedure was repeated seven times.

The use of brief labeling periods preferentially labels the viral DNA which is being synthesized in the cytoplasmic compartment. Infected cells were then loosened with the aid of a rubber policeman and collected by low-speed centrifugation, and the cell pellet was suspended in 1 ml of ice-cold water. An equal volume of ice-cold 10% trichloroacetic acid was added. The resulting precipitate was filtered onto Whatman GF/C filters, and its radioactivity was counted by liquid scintillation.

TABLE 1. Replication of individual VV α-A^r mutants[a]

Mutant no.	Derivation	Yield (PFU/dish)		% Resistance[b]
		+ α-amanitin	− α-amanitin	
WT		5.0×10^3	1.5×10^5	3.4
α-11	WT	1.5×10^4	8.3×10^4	18.0
α-14	WT	1.9×10^5	4.5×10^5	42.0
α-16	WT	7.4×10^5	2.1×10^6	35.66
α-27	HA	2.3×10^6	2.4×10^6	93.81
α-28	HA	1.5×10^4	5.0×10^4	30.0
α-30	HA	5.0×10^3	2.5×10^4	20.0
α-33	HA	$<10^3$	1.5×10^4	<6.6
α-34	HA	4.3×10^5	5.5×10^5	76.92
α-44	HA	5.0×10^4	2.5×10^5	20.0
α-46	HA	$<10^3$	5.3×10^4	<1.9
α-62	NG	2.7×10^5	1.8×10^6	14.65
α-63	NG	2.8×10^5	2.1×10^6	13.66
α-64	NG	1.2×10^5	1.2×10^6	9.4
α-69	NG	1.5×10^5	4.5×10^5	34.3
α-72	NG	9.3×10^5	1.8×10^6	50.68

[a] Monolayers of BSC-40 cells were infected in the presence or absence of 6 μg of α-amanitin per ml with WT VV or individually plaque-purified αA^r VV mutants. After 24 h, progeny virus was harvested, and titers were determined by plaque assay.

[b] (+ α-amanitin/− α-amanitin) × 100.

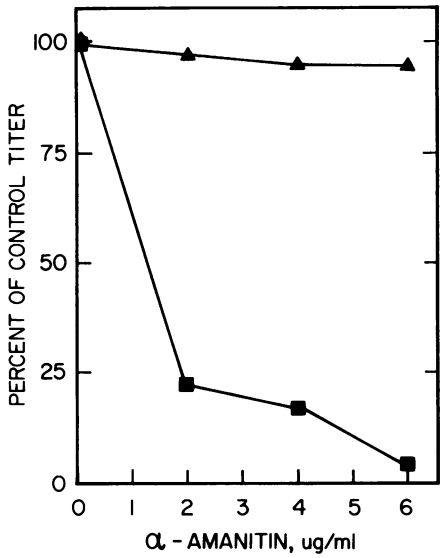

FIG. 2. Replication of α-27 (▲) versus WT (■) VV in the presence of increasing concentrations of α-amanitin. This experiment was carried out as described in the legend to Fig. 1. In the absence of drug the yields of infectious progeny per infected cell for WT and α-27 were 64.6 and 400, respectively.

(iv) Electron microscopy. Cells were infected with WT or α-27 VV at a multiplicity of 10 in the presence or absence of 6 μg of α-amanitin per ml. At 8 h postinfection, infected cell pellets were collected by centrifugation and suspended in 1 ml of 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4) containing 2 mM $CaCl_2$. After 2 min at 25°C, the suspension was centrifuged, the supernatant fluid was decanted, and the undisturbed pellets were fixed in the above glutaraldehyde solution for 30 min at 25°C. The pellets were postfixed in 1% osmium tetroxide in 0.15 cacodylate (pH 7.4) for 30 min and then stained en bloc with 0.5% uranyl acetate buffer (pH 5.0) for 30 min, dehydrated in acetone, and embedded in Epon 812. Thin sections were cut on a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate and lead citrate (18), and examined in a Hitachi HU-11E electron microscope at 80 kV.

Viral DNA extraction and gel analysis. Dishes (150 mm) of BSC-40 cells were infected at a multiplicity of 0.3 with WT or α-27 VV. After 72 h of infection, the infected cells were harvested, cytoplasmic extract was prepared, and the viral DNA was extracted and purified as previously described by Esposito et al. (7). Approximately 40 to 50 μg of viral DNA was obtained per dish of infected cells, and it was of sufficient purity for subsequent analyses. One microgram of WT or α-27 VV DNA was then digested with a variety of restriction endonucleases according to the conditions suggested by the manufacturer (Bethesda Research Laboratories). The resulting DNA fragments were resolved by electrophoresis at 50 V overnight in a 0.6% agarose (Seakem) gel in TAE (40 mM Tris-acetate [pH 8], 20 mM sodium acetate, 1 mM EDTA). The gels were stained with 0.5 μg of ethidium bromide per ml and photographed with a UV transilluminator and Polaroid type 57 film.

RESULTS

Isolation of α-A^r VV mutants. Before attempting the isolation of α-A^r VV mutants it was necessary to address two problems associated with this drug: slow uptake and generalized cellular toxicity. Previous experiments have shown that addition of α-amanitin to the nutrient medium at 18 h before infection is necessary and sufficient to equilibrate the effective dose (10). To determine the minimal concentration of α-amanitin that would suffice to inhibit VV replication (≥95%), the effect of increasing doses was titrated versus the ability of treated cells to support a VV infection. Addition of 6 μg of α-amanitin per ml to cells at 18 h before infection proved to

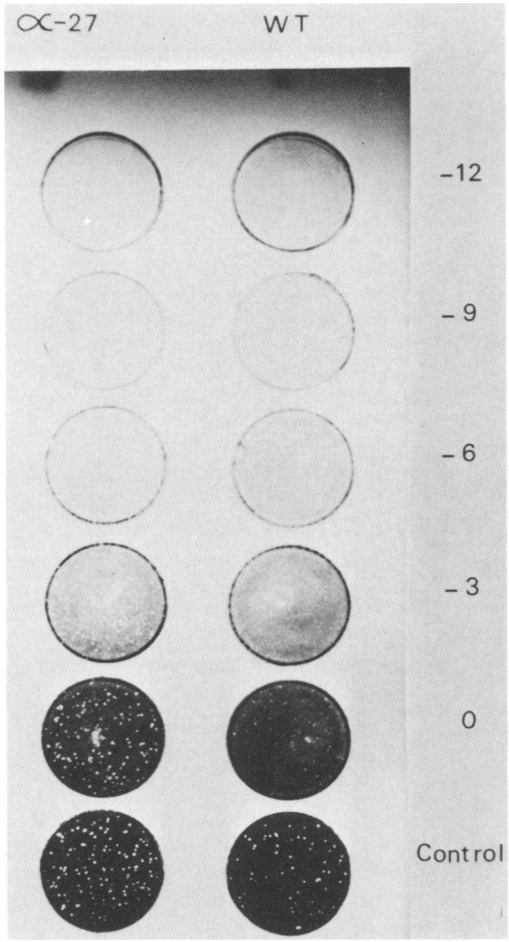

FIG. 3. VV plaque formation in the presence of α-amanitin. Before infection, BSC-40 monolayers were pretreated with α-amanitin for the indicated number of hours. At T_0 the monolayers were infected with ca. 50 to 100 PFU of α-27 or WT VV. After 48 h of infection in the presence of the drug, the monolayers were stained with crystal violet. Control dishes received no drug before or during infection.

TABLE 2. Replication of VV in enucleated BSC-40 cells [a]

Virus	Titer (PFU/60-mm dish)		%[b]
	Whole cells (×10^7)	Cytoplasts	
WT	1.17	3.05×10^5	2.6
α-27	4.64	1.74×10^7	37.5

[a] Enucleated (cytoplasts) or mock-enucleated (whole cells) BSC-40 cells were infected with WT or α-27 VV at a multiplicity of 5. After 24 h of infection, progeny virus was harvested, and titers were determined by plaque assay. Enucleation was carried out precisely as previously described by Hruby et al. (9).

[b] (Titer in cytoplasts/titer in whole cells) × 100.

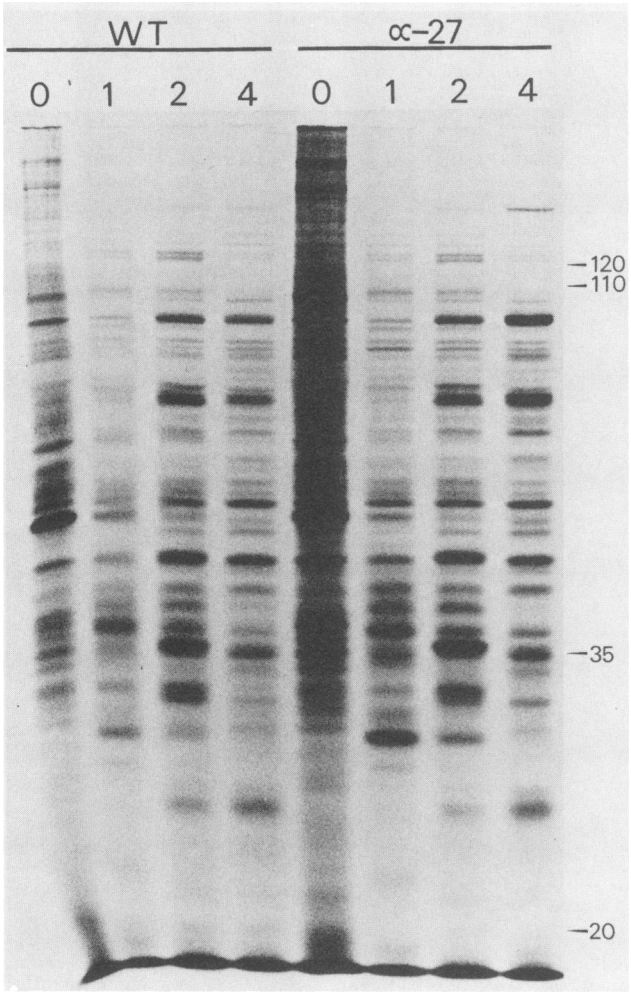

FIG. 4. α-27 and WT VV protein synthesis. BSC-40 cell monolayers were infected with α-27 or WT VV at a multiplicity of 25 in the presence of 6 μg of α-amanitin per ml. At the indicated hours postinfection, the cells were pulse-labeled for 30 min with 5 μCi of [^{35}S]methionine per ml. The infected cells were then collected, and the radioactively labeled proteins were analyzed by polyacrylamide gel electrophoresis and autoradiography. Numbers at the right indicate the molecular weights ($\times 10^3$) of marker polypeptides.

elicit a 95% inhibition of VV growth. Under these conditions, the cells maintained their normal morphology and exhibited high macromolecular biosynthetic capabilities (data not shown).

To select a α-A^r VV, low-multiplicity serial passages of WT, NG-mutagenized (NG-10), or HA-mutagenized VV were carried out in the presence of α-amanitin. Passages 1 to 3 resulted in a 3 to 4-log drop in the titer of infectious progeny (Fig. 1). However, beginning with passage 4, the emergence of α-A^r populations in each of the stocks became obvious. Later passages (5 to 7) allowed accentuation of the apparent α-A^r phenotype, resulting in the production of WT, HA, and NG crude stocks which had titers on the order of 10^6 PFU per dish. Since α-A^r progeny arose from each of the series after only four passages, this implies that the conversion to the drug-resistant phenotype was likely the result of a single mutation. The mathematical probabilities of simultaneously isolating multiple mutants in three separate passages are extremely low. Also, it should be noted that the α-A^r VV present after seven passages was likely to be a mixture of different mutants. These could be mutations in different genes or different mutations within the same gene. In either case, it was necessary to carry out plaque purification protocols before proceeding.

Twenty-five individual α-A^r plaques were picked from each of the three populations (WT, HA, NG). Of these, 30 were chosen at random and used to infect dishes of BSC-40 cells. The yield from each infection was titrated, and the 15 α-A^r mutants displaying the most growth were quantitatively tested for their ability to replicate in the presence or absence of α-amanitin (Table 1). Note that during the entire plaque purification scheme no drug was present. Therefore, if the α-A^r mutants had a high reversion frequency in the absence of selection, they had every opportunity to revert. As expected, the 15 α-A^r mutants displayed a variety of relative resistance to α-amanitin inhibition, ranging from 1.9% (α-46) to 50.7% (α-72) to 93.8% (α-27). There was no apparent correlation between the parental stock (WT, HA, or NG) and the level of α-amanitin resistance in the derived mutants.

For detailed analysis, we decided to concentrate initially on a single α-A^r VV mutant. On the basis of the data displayed in Table 1, α-27 was chosen. Its resistance to α-amanitin-mediated inhibition was assayed versus WT VV (Fig. 2). The replication of α-27 was essentially unaffected by concentrations of drug up to 6 μg/ml. Even at higher α-amanitin concentrations (8 to 10 μg/ml), α-27 still grew to 70 to 80% of control titers (data not shown). This is especially impressive considering the obvious cellular toxicity displayed at these drug doses. Another view of α-27 growth in the presence of α-amanitin is shown in Fig. 3. WT and α-27 VV were plaqued on BSC-40 monolayers which had been pretreated with α-amanitin for different lengths of time before infection. Although drug-mediated toxicity prevents plaque formation if the drug is added at 6 to 12 h before infection, there is a narrow time window 0 to 3 h before infection during which a differential effect can be seen. If α-amanitin was added at these times, control numbers of α-27 plaques were observed, whereas no or minute plaques were observed in the WT-infected dishes. These results confirm the α-A^r phenotype of α-27, and they indicate that single-step marker rescue experiments should be possible by assaying plaque formation of WT VV in the presence of α-amanitin added at −3 h and rescue with DNA fragments from α-27 DNA.

Biochemical analysis of α-27 growth. As an initial step toward discerning the nature of the α-27 mutation which enables VV to replicate in the presence of α-amanitin, several facets of virus growth were compared biochemically between α-27 and WT VV. One of the most easily measured parameters of a VV infection is cytoplasmic DNA synthesis. The kinetics and amount of α-27 DNA synthesis were indistinguishable from those of WT when analyzed by pulse-labeling with [^{3}H]thymidine. Both displayed a sharp peak of incorporation at 2 h which decreased thereafter (data not shown). Viral gene expression was analyzed by pulse-labeling infected cells at various times postinfection with [^{35}S]methionine. The qualitative and quantitative expression of α-27 viral proteins looked identical to that of WT at both early and late times postinfection (Fig. 4). Of course, this is only a one-dimensional analysis, and a two-dimensional gel might possibly elucidate more subtle differences. In short, no differences could be observed between α-27 and WT VV at the levels of DNA, RNA, or protein synthesis.

Replication of α-27. Previous electron micrographs of VV-infected cytoplasts have shown an accumulation of imma-

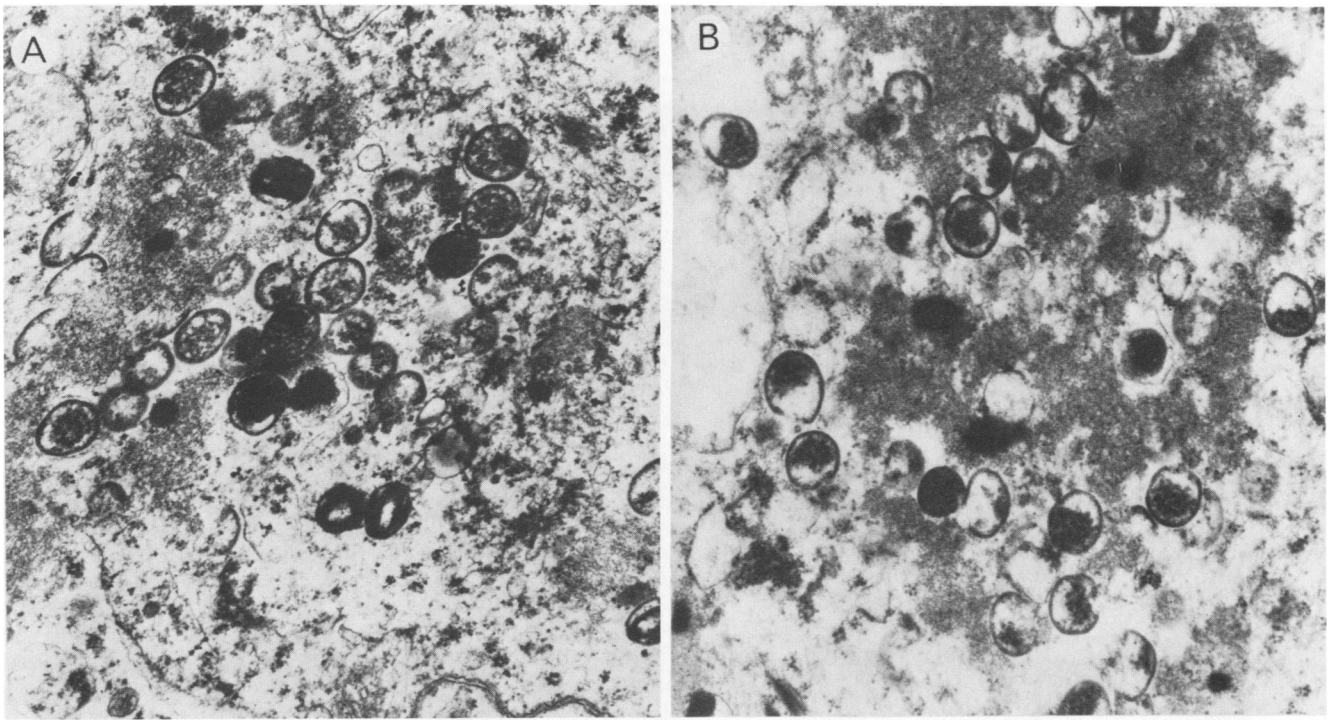

FIG. 5. Electron micrographs of VV morphogenesis. Cells were infected with WT (A) or α-27 (B) VV at a multiplicity of 10 in the presence of α-amanitin. At 8 h postinfection, the infected cells were harvested, fixed with glutaraldehyde, thin sectioned, and then examined by electron microscopy.

ture morphogenetic forms which were apparently arrested at a specific stage in development (9). Since VV replication is identical biochemically in cytoplasts and α-amanitin-treated cells, it has been presumed that virus development is aborted at the same stage. However, this has not been examined directly. Figure 5 shows electron micrographs of α-27- and WT-infected BSC-40 cells at 8 h after infection in the presence of α-amanitin. Electron-dense viroplasm is quite obvious in both situations, indicating viral DNA and protein synthesis. The α-27 infection shows a variety of developmental forms including immature particles, particles with condensing DNA (eyeballs), and mature brick-shaped virions. The WT infection, however, is arrested at a point similar to that previously described for enucleated cells, namely, viral membranes form around viral DNA and protein (viroplasm), but this structure does not coalesce and condense into a recognizable virus particle.

The immature particles seen in Fig. 5B are reminiscent of those which accumulate in the presence of rifampin. Rifampin is a specific inhibitor of VV replication. It acts, either directly or indirectly, by inhibiting the cleavage of the two major virion precursor polypeptides, P94 and P65, into the mature VP62 and VP60 core proteins (12, 14). The modes of rifampin- and α-amanitin-mediated inhibition are apparently distinct though, as α-27 was unable to replicate in rifampin-treated cells (data not shown). However, since the effects of α-amanitin and rifampin were visibly similar, we decided to see if they were biochemically analogous, namely, inhibiting VV-specific proteolytic cleavages. In Fig. 6 are shown the results of a protein pulse-chase experiment carried out at late times postinfection with either α-27 or WT VV in the presence of α-amanitin. A most surprising result is evident. WT VV, in the presence of the drug, synthesized p94 and p65 but was unable to process them into VP62 and VP60. In contrast, the processing of α-27 core polypeptides occurred normally in the presence of α-amanitin.

Earlier, it was stated that replication of VV was blocked at the same stage in enucleated, UV-irradiated, or α-amanitin-treated cells. If this statement is correct, then one might predict that α-27 should be capable of growth in enucleated cells. To test this, monolayers of BSC-40 cells were enucleated, or mock enucleated, with cytochalasin B. After a recovery period, the monolayers were infected with either α-27 or WT VV. After 24 h, the infectious progeny were harvested, and titers were determined (Table 2). In agreement with previous results (9), WT VV was unable to grow in the absence of the host cell nucleus. The low levels of infectious progeny produced are likely the result of the remaining 2 to 3% nucleated cells. In contrast, α-27 grew to 37.5% of control titer in enucleated cells. Considering the insult to the integrity of the cells due to the enucleation procedure, this was a quite appreciable level of replication. In fact, α-27 replicates better in cytoplasts than do truly cytoplasmic viruses such as poliovirus or reovirus (8).

Genomic structure of α-27. As a first step toward determining the nature of the α-27 mutation, it was necessary to examine the genomic α-27 DNA to make sure no large deletions, insertions, or sequence alterations had occurred during the mutant isolation procedure. To this end, DNA was extracted and purified from α-27 and WT VV. The viral DNA was digested with a variety of restriction endonucleases and analyzed by agarose gel electrophoresis. The typical result is shown in Fig. 7A, in which the *Hin*dIII digests of α-27 and WT DNA are indistinguishable, indicating the absence of any sizeable sequence alterations. The same results were obtained when the enzymes *Sst*I, *Pst*I, or *Sal*I were used (data not shown). However, when *Xho*I digests of α-27 and WT DNA were compared (Fig. 7B), a

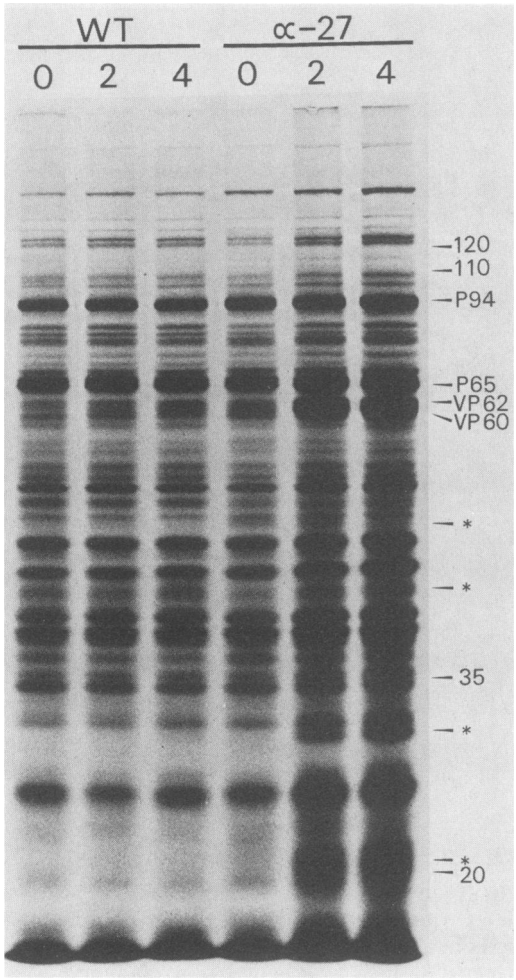

FIG. 6. α-Amanitin-mediated effects of VV proteolytic cleavages. Cell monolayers were infected with α-27 or WT VV at a multiplicity of 25 in the presence of α-amanitin. At 5 h postinfection, the infected cells were pulse-labeled for 30 min with 5 μCi of [^{35}S]methionine per ml. Isotope-containing medium was then replaced with medium containing unlabeled methionine (×100), and infection was allowed to proceed for the indicated number of hours. Infected cell proteins were analyzed by polyacrylamide gel electrophoresis and autoradiography. The two major VV core proteins, VP62 and VP60, and their precursors, P94 and P65, are indicated. Other proteins which accumulated during the chase period are marked with an asterisk. The numbers at the right indicate the molecular weights (×10^3) of marker proteins.

difference emerged. It can be seen that in the α-27 DNA *Xho*I digest, the 3.1-kilobase (kb) *Xho*I O fragment is missing. In its place is a larger 24-kb fusion fragment resulting from the joining of the 3.1-kb *Xho*I O and 21.9-kb *Xho*I C fragments. The identity of the α-27 O-C fusion fragment was confirmed by the subsequent subdigestions (data not shown). In the lower portion of Fig. 7 the appropriate portions of the VV *Hin*dIII, *Xho*I (6), and *Sst*I (Hruby, unpublished data) maps are drawn and aligned so the approximate location of this alteration in the α-27 genome can be determined.

DISCUSSION

In retrospect, the decision to attempt the isolation of α-A^r VV mutants may have been imprudent. If either of the two explanations for the nuclear requirement in VV replication, namely, cellular transcription of viral genes or host factor catalysis of viral assembly, were correct, then it is likely that a number of viral loci would be involved. Therefore, the isolation of a VV mutant capable of circumventing the host cell nucleus would likely require a number of alterations. The probability of selecting such a multiple mutant is extremely low. However, as the data in Fig. 1 demonstated, α-A^r VV mutants were readily selected from both WT and mutagenized viral stocks. These mutants were quite stable and did not revert back to an α-amanitin-sensitive state even after multiple passages in the absence of drug selection. The ease and rapidity with which the α-A^r phenotype arose implies that only a single mutation was required. As such, it is unlikely that either of the previously offered hypotheses are entirely correct.

In all, 75 individual α-A^r VV mutants were isolated and plaque purified. The experiments reported here have centered on a single mutant, α-27, whose replication was essentially unaffected by 6 μg of α-amanitin per ml. Comparative biochemical analyses between α-27 and WT VV failed to discern any differences in viral gene expression or DNA synthesis, in either the presence or the absence of the drug. However, pulse-chase experiments revealed that α-27 was able to mature its two major core precursors, P94 and P65, into VP62 and VP60 in the presence of α-amanitin whereas WT was not. The block in this obligatory processing reaction by α-amanitin was confirmed by electron microscopy, which showed an accumulation of immature WT VV developmental forms when the drug was present. It should not be assumed, though, that the primary effect of α-amanitin inhibition is to block this proteolytic step. The morphogenesis of VV is a very complex procedure involving a large number of steps. If one of the initial steps is aberrant, then perhaps the assembly process never progresses to the configurational state necessary to allow processing. Thus, the α-27 mutation might very well affect an earlier step in replication which is only seen late in infection.

The ability of α-27 to replicate in enucleated cells was both surprising and remarkable. If one considers that after enucleation there is essentially a bag of cytoplasm left, then upon VV infection the virus is able to specify all the information necessary to transcribe its many genes, regulate their expression, replicate its DNA, and catalyze its own assembly. All in all, perhaps the original notion of poxviruses as cytoplasmic-replicating entities was not so farfetched after all. Recently, Archard (1) has described the synthesis of VV DNA within isolated HeLa cell nuclei. This result is in line with those of Wing and Weissbach (24), who have reported finding VV RNA polymerase associated with the host cell nucleus and have suggested that VV transcribes some of its RNA within the nucleus of infected cells. If so, the ability of α-27 to replicate in cytoplasts would indicate that this is not an obligatory step in the VV life cycle.

It is doubtful whether additional biochemical analyses of α-27 will further enlighten us as to the mechanism by which this mutant circumvents α-amanitin-mediated inhibition. We are therefore in the process of trying to map the location of this mutation on the VV genome so that we can carry out more definitive experiments. Two approaches are being taken to map the α-27 mutation. First, we have selected a number of temperature-sensitive α-A^r VV mutants. If, by recombinational analyses, we can find a mutant whose dual phenotype is the result of a single or two closely linked mutations (21), then we can map the genomic location of the α-A^r temperature-sensitive mutation by complementation analysis with other VV mutants (5). The second approach we

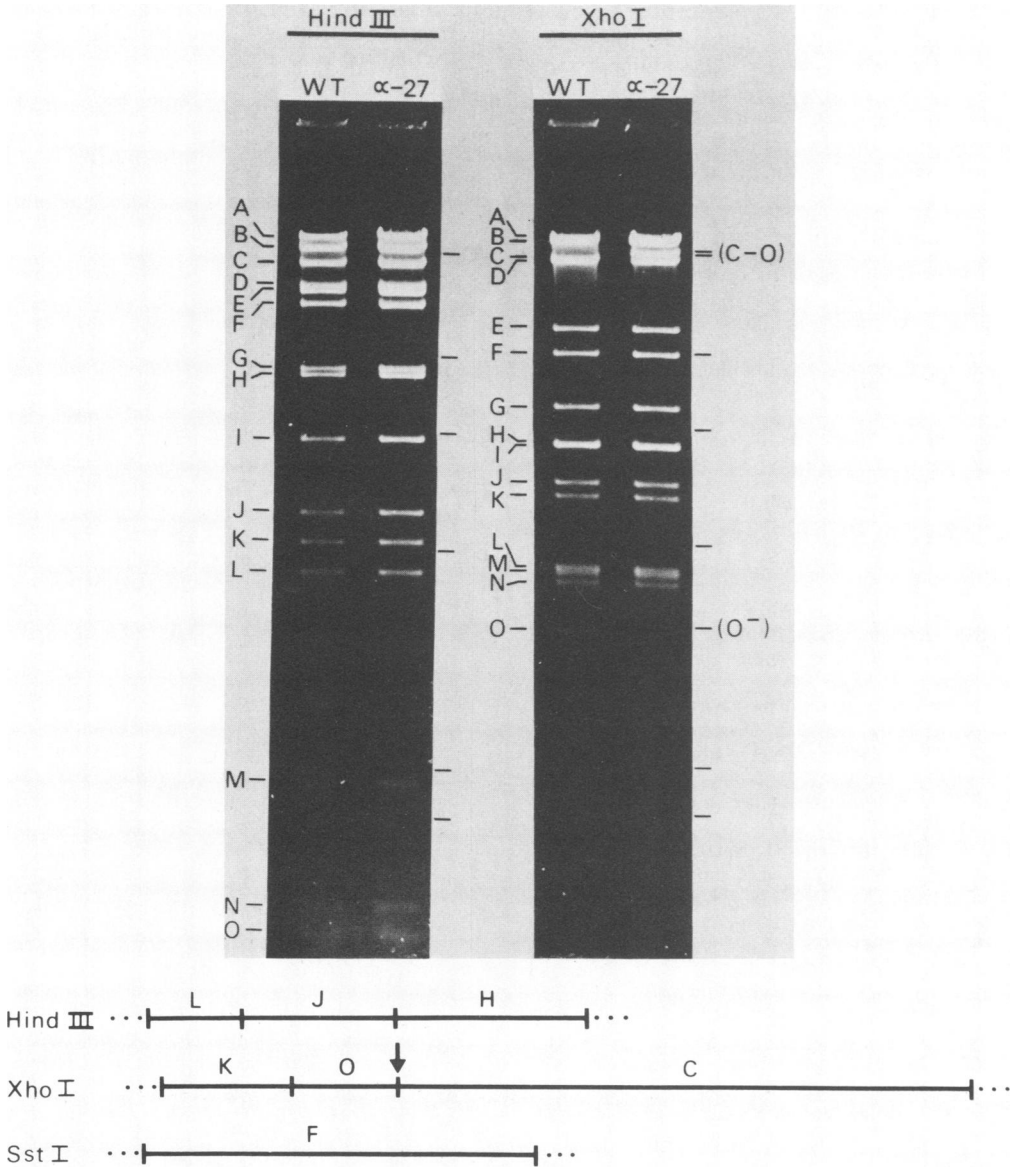

FIG. 7. Restriction enzyme analysis of α-27 and WT VV DNA. Purified α-27 and WT VV DNA was restricted with either *Hin*dIII or *Xho*I, and the resulting fragments were resolved by agarose gel electrophoresis. The VV DNA bands are lettered by size, according to convention. The markers on the right side of the gels indicate the positions of the fragments from a λ DNA *Hin*dIII digest. *Hin*dIII, *Xho*I, and *Sst*I restriction maps of the portion of the VV genome of interest are aligned and shown below the gels. The arrow indicates the *Xho*I cleavage site missing in α-27 VV DNA.

have taken is to rescue the growth of WT VV by transfecting the infected monolayers with all, or parts, of the α-27 genome. Toward that goal we performed a restriction enzyme analysis of the α-27 genome and discovered that the *Xho*I cleavage site between the O and C fragments had been altered. Of course, finding a mutated restriction site was fortuitous, but given that α-27 is derived from an HA stock (HA catalyzes the conversion of G-C → A-T base pair changes) and that the recognition sequence of *Xho*I is CTCGAG, it is perhaps not totally unexpected. Furthermore, the genome size of VV is on the order of 185 kb, so finding a single altered enzyme cleavage site certainly doesn't prove that this is the site of the α-27 mutation. However, in preliminary experiments, we have been able to efficiently rescue WT VV with intact, *Xho*I- or *Sst*I-cleaved α-27 DNA, whereas *Hin*dIII-restricted α-27 DNA was much less effective. If *Sst*I fragment F from α-27 DNA was preparatively isolated from gels and used in the marker rescue assay, it too efficiently rescued the WT VV. Taken together, this information is suggestive that the α-27 mutation lies very close to the *Hin*dIII J-H junction. It will be of interest to ascertain whether other α-A^r VV mutants such as α-34, α-14, and α-72 also map to this region. One large VV early transcript is known to originate from this region (2), as well as several late transcripts (Hruby, unpublished data). Whether any of these transcripts encode a protein involved with α-amanitin inhibition of growth remains to be seen.

We are currently in the process of subcloning the α-27 genome to precisely map the location of the α-27 mutation. Once this is done we hope to use the cloned fragments to

identify the transcript and encoded polypeptide which is responsible for α-amanitin resistance. Once identified, this protein will be used to raise monospecific antisera with which to follow its synthesis and to identify the viral and host polypeptides that it associates with. It is hoped that such efforts will shed light on new facets of VV replication and how this virus interacts with the host cell during infection.

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