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Mapping the Genomic Location of the Gene Encoding α-Amanitin Resistance in Vaccinia Virus Mutants†

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To facilitate the determination of the genomic location of the vaccinia virus gene(s) encoding α-amanitin resistance (α^r) (Villarreal et al., J. Virol. 51:359–366, 1984), a collection of α^r, temperature-sensitive (ts) mutants were isolated. The premise of these experiments was that mutants might be found whose dual phenotypes were the result of a single or two closely linked mutations. Genetic analyses of the α^rts mutant library revealed two mutants, α^rts7 and α^rts12, that apparently fit this criterion; in α^rts7 the two lesions were indistinguishable, whereas in α^rts12 the two mutations were closely linked but separable. Cloned vaccinia virus *Hin*dIII DNA fragments were used to marker rescue the temperature-sensivite phenotype of these two dual mutants. The temperature-sensitive lesion of α^rts7 was rescued by the *Hin*dIII N fragment (1.5 kilobases), whereas α^rts12 was rescued by the neighboring *Hin*dIII M fragment (2.0 kilobases). The progeny virions of the α^rts7 *Hin*dIII-N rescue reverted to an α-amanitin-sensitive phenotype, whereas the α^rts12 *Hin*dIII-M progeny were still resistant to the drug. Taken together, these data indicate that the gene encoding α-amanitin resistance maps to the *Hin*dIII N fragment and provides evidence for the existence of essential vaccinia virus genes in a region of the genome previously believed to be nonessential for replication in tissue culture. Biochemical analyses revealed that both mutants were capable of synthesizing DNA as well as early and late viral proteins at the permissive and nonpermissive temperatures. At the nonpermissive temperature α^rts12 and α^rts7 were unable to process the major core precursors P94 and P65 into VP62 and VP60.

Poxviruses, such as vaccinia virus (VV), are believed to be capable of replicating and transcribing their viral DNA entirely within the cytoplasmic compartment of susceptible host cells (3, 12, 13). Since VV virions contain or encode many, if not all, of the enzymes required for the replication and expression of their genetic information, this led early investigators to postulate that VV replicated solely in the cytoplasm of infected cells, completely independent of the host cell nucleus (12). This notion was challenged in 1974 by the experiments of Pennington and Follett (15), which showed that VV was unable to complete its replicative cycle in cells that had been subjected to cytochalasin B-mediated enucleation before infection. This initial observation concerning the existence of some essential interaction between VV and the host cell nucleus has been followed up by a series of experiments which have led to the conclusion that active participation of the host cell transcriptional apparatus is required for the production of infectious VV progeny. The most convincing argument in this regard was the discovery that VV replication is blocked by the drug α-amanitin (10 μg/ml), a potent inhibitor of host but not viral transcription (9, 16, 17), implicating cellular RNA polymerase II as the target.

As an initial effort toward understanding the nature of the viral as well as the host cell involvement in this interaction, a VV mutant (α-27) which was capable of replicating in the presence of α-amanitin was selected and isolated (20). A biochemical analysis of α-27 replication revealed no differences, either in the presence or absence of α-amanitin, when compared with wild-type VV with respect to DNA synthesis or viral protein synthesis. However, there was a marked difference in the ability of the two viruses to direct the proteolytic processing of the two major core precursor polypeptides, P94 and P65, in the presence of the drug. This processing reaction was completely blocked by α-amanitin in wild-type (WT) VV-infected cells, but proceeded normally in α-27-infected cells. It was not clear whether the ability to carry out this cleavage reaction in the presence of α-amanitin represented the primary, or a secondary, effect of the α-27 mutation. In either case, it was unclear how to proceed with this problem without first identifying the viral gene product(s) that were involved. Thus it became necessary to determine the genomic location of the mutation giving rise to the α-amanitin-resistant (α^r) phenotype so that viral proteins encoded by this region could be more closely scrutinized as to their structure and function.

We report here the results of applying genetic mapping approaches to determining the genomic position of the α^r locus of VV. These data have allowed the gene encoding this function to be localized within the 1.5-kilobase (kb) *Hin*dIII N fragment and provide the first evidence of an essential complementation group to be located in the region. These results are discussed in the context of other recent work (6) with respect to the identity of the gene product encoded at this site.

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 gentamycin sulfate per ml. Parental VV (WR strain) was obtained from the American Type Culture Collection in 1977 and has been propagated by low-multiplicity passages and periodic plaque purification ever since. Hydroxylamine-mutagenized VV was prepared as previously described and kindly provided to us by R. C. Condit (2). Viral infections and

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plaque assay titrations were performed as previously described (8).

Isolation of α^r, temperature-sensitive (ts) mutants. Confluent monolayers of BSC-40 cells (60-mm dishes) were pretreated with 6 μg of α-amanitin (Sigma Chemical Co.) per ml for 18 h before infection to equilibrate the effective drug dosage (9). The pretreated cells were then infected at a multiplicity of 1 with the hydroxylamine-mutagenized stock 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. Infectious titers were determined, and this crude preparation of virus was used to infect a second set of α-amanitin-pretreated BSC-40 cells. This set of steps was repeated a total of six times. All manipulations and incubations were done at the permissive temperature, 31°C (2).

After six serial passages, a drug-resistant virus population had arisen from the parental hydroxylamine-mutagenized stock. Individual α^rts mutants were obtained by use of a plaque enlargement technique (18). Appropriate dilutions of the drug-resistant virus were plated under a nutrient agar overlay at 31°C. After 3 days of incubation, dishes were overlaid with nutrient agar containing 0.005% neutral red. The following day, plaque sizes were marked on the back of the dish by filling in the plaques with a felt-tipped pen. Plates were then incubated at 40°C. Three days later, plaques which had not enlarged were picked with sterile Pasteur pipettes and suspended in 2 ml of phosphate-buffered saline containing 2% fetal calf serum. Virus was released from infected cells by one cycle of freeze-thawing (18). Individual mutants were then tested for their temperature sensitivity and resistance to α-amanitin as described previously (20), except that incubation of infected cells was carried out at 31°C. The screening procedure mentioned above yielded a number of temperature-sensitive, α-amanitin-resistant mutants, two of which (α^rts7 and α^rts12) displayed sufficient temperature sensitivity and drug resistance to be of use in further studies.

Genetic mapping. Quantitative recombination and complementation analysis between α^rts7 and α^rts12 were carried out as described previously by Condit and Motyczka (2).

To determine the recombination frequency between the two phenotypes (α^r and ts) of each mutant, BSC-40 cells were infected at a multiplicity of 6 each with wild-type virus and α^rts7 or α^rts12, or at a multiplicity of 12 with either mutant alone. After the unadsorbed virus was removed and minimal essential medium containing 10% fetal calf serum was added, the infected cells were incubated at 31°C for 2 days. The progeny virions were harvested, titered, and passaged in the presence of 6 μg of α-amanitin per ml at 40°C. Virus yields were determined by plaque titration, and the recombination frequencies were calculated (2).

The leakiness test was carried out as described previously by Condit and Motyczka (2). To determine whether the two phenotypes of each mutant were the result of one or two mutations, backcrossing experiments between α^rts7 (or α^rts12) and WT VV were carried out as described by Sridhar and Condit (19).

Marker rescue. Recombinant DNA plasmids containing the WT VV *Hin*dIII DNA fragments were linearized by digestion with the restriction enzyme *Hin*dIII. The DNAs were then purified by extraction with TE (10 mM Tris chloride [pH 8], 1 mM EDTA)-saturated phenol, extracted with H_2O-saturated ethyl ether, and ethanol precipitated twice. These manipulations essentially sterilized the DNA molecules. The plasmid DNAs were then suspended in sterile H_2O and coprecipitated with salmon sperm DNA to a final concentration of 20 μg of total DNA per ml by the calcium phosphate method originally described by Graham and Van der Eb (7). Confluent 60-mm dishes of BSC-40 cells were infected with the α^rts mutant viruses at a multiplicity of 0.1 PFU/cell. After an hour of adsorption at 31°C the viral inoculum was removed, medium was added to the monolayers, and the infected cells were incubated at 31°C to allow the early event in viral infection to be initiated. After 4 h, the medium was removed, the monolayers were treated with 15% glycerol for 40 s (19), and the DNA precipitates were added to the cells. The infected cells were incubated at 40°C for 3 h, the medium containing the precipitated DNA was then removed, the monolayers were washed once with fresh medium, 4 ml of medium was added to the dishes, and they were incubated at 40°C. After 48 h the viral progeny were harvested and titered at both the permissive (31°C) and nonpermissive temperatures (40°C).

Cloning. The cloned WT *Hin*dIII fragments used in the marker rescue experiments were originally obtained from B. Moss (1). All other recombinant DNA manipulations were carried out essentially as described by Maniatis et al. (10).

Biochemical analyses. Early and late viral gene expression of both mutants and WT VV at the permissive and nonpermissive temperature was analyzed as previously described by Villarreal et al. (20).

To examine viral DNA synthesis, BSC-40 monolayers were infected with virus at a multiplicity of 10 and incubated at 31 or 40°C. At various times postinfection the infected monolayers were harvested, washed twice with phosphate-buffered saline, and suspended in 1 ml of phosphate-buffered saline. The cells were then lysed by three freeze-thaw cycles, treated with 0.25% trypsin for 30 min at 37°C, and collected on a nitrocellulose sheet by filtration with a microsample manifold (Schleicher & Schuell Co.). The filter was blotted three times on successive Whatman 3MM paper saturated with (i) 0.5 M NaOH and (ii) 1 M Tris hydrochloride (pH 7.6) and washed for 15 min in 0.1 M Tris hydrochloride (pH 7.6)–0.15 M NaCl. The filter was baked at 80°C for 2 h and then incubated with 5× Denhardt solution, 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 50% deionized formamide, and 100 μg of denatured salmon sperm DNA per ml for 4 h. The filter was then hybridized in a mixture consisting of 6× SSC, 5× Denhardt solution, 10× dextran sulfate, 100 μg of denatured salmon sperm DNA per ml, 50% deionized formamide, and DNA labeled with ^{32}P by nick translation (cloned *Hin*dIII J fragment) for 12 h. The filter was washed three times at 42°C for 30 min in 2× SSC–0.1% sodium dodecyl sulfate and three times at 50°C for 30 min in 0.2× SSC–0.1% sodium dodecyl sulfate, dried, and placed on Kodak XAR-5 X-Omat film at −70°C.

RESULTS

Our initial efforts to determine the genomic location of the VV α^r gene were directed toward using the drug-resistant phenotype to rescue the replication of WT VV from α-amanitin-induced inhibition. To carry out these experiments, the *Hin*dIII and *Xho*I fragments of the DNA genome of the α^r mutant α-27 were subcloned into both plasmid and cosmid vectors. However, when these fragments were used in marker rescue experiments, it was discovered that the combination of the α-amanitin-mediated toxicity and the transfection of the calcium phosphate-precipitated DNA rendered the recipient BSC-40 cells essentially incapable of supporting a viral infection. Although low levels of pheno-

typic rescue were observed, two- to threefold over background (data not shown), it did not appear that this level of signal strength would provide an adequate assay for further mapping studies. Therefore as an alternative approach, we sought to obtain a collection of α^rts mutants in the hopes that one or more of these mutants would display dual phenotypes that were the result of single or two closely linked mutational events. If such were the case, then a variety of well-established genetic mapping procedures could be employed to locate the site of the α^rts lesion (2, 4, 5).

To select α^rts mutants, essentially the same protocol was employed as that previously used to isolate α-27 (20), namely, low-multiplicity serial passages of hydroxylamine-mutagenized VV were carried out in the presence of 6 μg of α-amanitin per ml, except the entire procedure was carried out at the permissive temperature of 31°C. Passages 1 and 2 resulted in a 3-log drop in the titer of infectious viral progeny. The emergence of an α^r population became obvious in passage 3 and became amplified by passages 4 through 6 (data not shown). Potential α^rts mutants were initially identified from the progeny of passage 6 by using a plaque-enlargement technique, which allowed large numbers of plaques to be screened (2). Sixty individual plaques were isolated and grown up. Each was subjected to (i) a spot test to confirm its ts phenotype (2) and (ii) a quantitative determination of the degree of resistance to α-amanitin inhibition. It appeared that 11 of the 60 plaque isolates possessed the desired dual phenotypes (Table 1). Of these 11, only 2 (α^rts7 and α^rts12) were judged to be sufficiently drug resistant to be of interest. α-Amanitin resistance (ratio of efficiency of plating at 31°C with α-amanitin to that without α-amanitin) was 55% for α^rts7 and 40% for α^rts12. Therefore, these two mutants were selected for further genetic analyses.

Genetic analyses. Before proceeding any further with the genetic and mapping analyses of the two isolated mutants (α^rts7 and α^rts12), it was important to determine the degree of leakiness of each mutant. The leakiness test [(one-step growth at 40°C/one-step growth at 31°C) × 100] yielded values of 0.2 and 0.17% for α^rts7 and α^rts12, respectively. As discussed above, the usefulness of an α^rts mutant for mapping purposes depends on the two phenotypes (α^r and ts) being the result of one or two closely linked mutations. To address this question, a backcross was carried out at the permissive temperature (31°C) between WT VV and either α^rts7 or α^rts12. The progeny of the backcross was passaged in the presence of α-amanitin at the nonpermissive temperature (40°C), and the progeny virions from this step were harvested and plaque titrated at 40°C. If α-amanitin resistance and temperature sensitivity result from two different mutations, recombination with WT virus between the mutations will cause the phenotypes to segregate into progeny which are only α^r and only ts. The α^r mutants will produce a higher titer at 40°C in the presence of the drug than that of the parental viruses (19). In the case of α^rts7 the titer was not higher than that of the parents, whereas that of α^rts12 was higher than that of the parental strains (Table 2). Recombination frequencies between the α^r and ts mutations of α^rts7 and α^rts12 yielded values of 0.7 and 1.4% respectively. Previous workers have shown that recombination frequency between two noncomplementing VV ts mutants is usually less than 1% (2, 4, 5, 19). These results tend to indicate that in the case of α^rts7, α^r and ts result from a single or two very closely linked mutations within the same gene, whereas in the case of α^rts12 they are the result of two distinct but closely linked mutations.

TABLE 1. Screening mutants for temperature sensitivity and α-amanitin resistance

Mutant no.	Temperature sensitivity	$\alpha^{r a}$ (%)
7	+	55
12	+	40
20	+	2
30	+	4.5
37	+	10
40	+	3
42	+	4.8
46	+	5.3
50	+	9
59	+	2
60	+	4.1

[a] Efficiency of plating (31°C) with α-amanitin/efficiency of plating (31°C) without α-amanitin. Only mutants with dual phenotypes are displayed. The other 49 mutants failed to demonstrate a temperature-sensitive phenotype in the spot test.

TABLE 2. Genetic characteristics of α^rts7 and αts12[a]

Cross	Selective conditions for assay of virus yield	Recombination frequency (%)	Titer (PFU/ml)
Cross			
α^rts7 × WT	40°C, α-amanitin	0.7	
α^rts12 × WT	40°C, α-amanitin	1.4	
α^rts7 × α^rts12	40°C	12	
Backcross			
α^rts7 × WT	40°C, α-amanitin		1.96×10^5
WT × WT	40°C, α-amanitin		3.23×10^5
α^rts7 × α^rts7	40°C, α-amanitin		1.02×10^5
α^rts12 × WT	40°C, α-amanitin		9.6×10^4
WT × WT	40°C, α-amanitin		8.7×10^4
α^rts12 × α^rts12	40°C α-amanitin		3.9×10^4

[a] The complementation index of α^rts7 for the α^rts7 × α^rts12 cross was 2.9.

Marker rescue. Since ts and α^r lesions appeared to be closely linked in α^rts7 and α^rts12, it seemed that a close approximation of the genomic location of the gene encoding α-amanitin resistance could be obtained by mapping the ts mutations of these two dual mutants. Therefore, the ability of cloned WT VV *Hin*dIII DNA fragments (D through O) as well as the *Hin*dIII C fragment preparatively isolated from gels to rescue the replication of α^rts7 or α^rts12 at the nonpermissive temperature (40°C) was assessed (Table 3). The *Hin*dIII A and B fragments, which due to their large size have not been cloned in our laboratory, were not used in the marker rescue experiments. In the case of α^rts7, the transfection of plasmid DNA, carrier DNA, or 13 of the individual VV *Hin*dIII DNA fragments did not provide the necessary functional gene to rescue out the ts lesion. The *Hin*dIII N fragment, however, proved most efficient in this regard. In the case of α^rts12 a similar situation existed, except that this mutant was rescued by the neighboring *Hin*dIII M fragment. One of the predictions of the two phenotypes arising from the same or two closely linked mutations is that the DNA fragment that rescues the ts phenotype should also convert the α^r phenotype back to WT, namely, α^s. The progeny virions from the α^rts7–*Hin*dIII-N and α^rts12–*Hin*dIII-M rescues were assayed for their resistance to α-amanitin-mediated inhibition. The N fragment converted α^rts7 to both temperature insensitivity and drug sensitivity (1% resistance), whereas the progeny of the α^rts12–*Hin*dIII-M rescue retained their drug-resistant character (55% resistance).

TABLE 3. Marker rescue of α^rts7 and α^rts12 by using *Hin*dIII fragments from WT VV

Expt	*Hin*dIII fragment	Titer (PFU/ml), 40°C		Titer (PFU/ml), 31°C	
		α^rts7	α^rts12	α^rts7	α^rts12
I	C	0	0	5.0×10^3	3.7×10^3
	D	0	0	5.2×10^3	3.0×10^3
	E	0	0	4.8×10^3	4.1×10^3
	F	0	0	4.3×10^3	6.0×10^3
	G	0	0	4.8×10^3	7.0×10^3
	H	0	0	7.2×10^3	4.5×10^3
	I	0	0	6.0×10^3	5.5×10^3
	J	0	0	6.6×10^3	2.0×10^3
	K	0	0	4.7×10^3	6.3×10^3
	L	0	0	6.0×10^3	4.6×10^3
	M	0	4.6×10^3	8.0×10^3	4.2×10^3
	N	6.0×10^3	0	5.5×10^3	5.0×10^3
	O	0	0	1.0×10^4	1.0×10^3
	pBR322	0	0	6.2×10^3	3.0×10^3
	Salmon sperm DNA	0	0	6.3×10^3	6.0×10^3
	None	0	0	5.4×10^3	3.5×10^3
II	C	0	0	3.8×10^4	5.3×10^4
	D	0	0	3.24×10^4	4.8×10^4
	E	0	0	3.4×10^4	3.2×10^4
	F	0	0	5.0×10^4	5.0×10^4
	G	0	0	3.5×10^4	3.0×10^4
	H	0	0	3.7×10^4	3.7×10^4
	I	0	0	4.3×10^4	1.0×10^4
	J	0	0	2.7×10^4	2.7×10^4
	K	0	0	5.0×10^4	6.3×10^4
	L	0	0	2.5×10^4	2.8×10^4
	M	0	5.0×10^4	2.0×10^4	4.0×10^4
	N	4.0×10^4	0	3.6×10^4	3.0×10^4
	O	0	0	1.0×10^4	3.0×10^4
	pBR322	0	0	3.3×10^4	5.0×10^4
	Salmon sperm DNA	0	0	3.6×10^4	5.3×10^4
	None	0	0	2.6×10^4	3.2×10^4

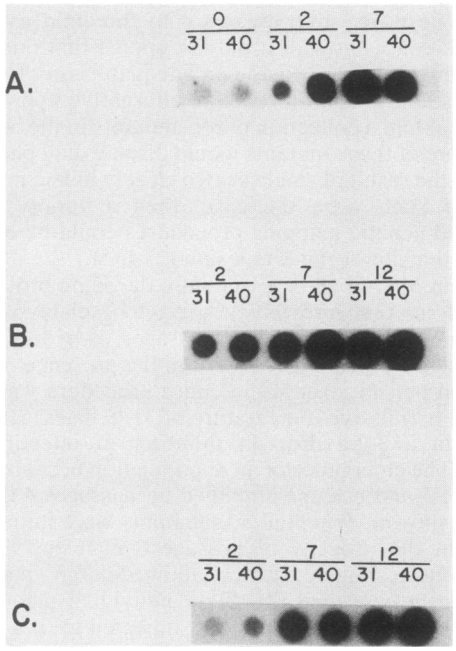

FIG. 2. Dot-blot hybridization of viral DNA from BSC-40-infected cells. Duplicate plates of BSC-40 cells were infected with either the WT, α^rts7, or α^rts12 at a multiplicity of 10 and incubated at 31 and 40°C. At various times postinfection the infected monolayers were harvested, and the viral DNA was extracted and used for the dot-blot hybridization assay described in Materials and Methods. The ^{32}P-labeled *Hin*dIII-J DNA probe was prepared from cloned VV DNA (1) by the method of Maniatis et al. (10). The specific activity of the nick-translated probe was 1.5×10^8 cpm/μg. (A) DNA extracted from WT-infected cells at 0, 2, and 7 h postinfection after incubation at 31 and 40°C. (B) DNA extracted from α^rts7-infected cells at 2, 7, and 12 h postinfection after incubation at 31 and 40°C. (C) DNA extracted from α^rts12-infected cells at 2, 7, and 12 h postinfection after incubation at 31°C and 40°C.

These results are consistent with the complementation and recombination experiments (Table 2), which suggested that the ts7 and ts12 mutations were in closely linked but different genes. Figure 1 shows the genomic location of both mutations in the context of the VV *Hin*dIII restriction map.

It was of interest to examine the biochemistry of α^rts7 and α^rts12 replication to see how it correlated with previous observations with VV α^r mutants (20). Therefore, viral DNA and protein synthesis at the permissive (31°C) and nonpermissive (40°C) temperatures as well as proteolytic cleavage of the major core precursors were analyzed in the hope of obtaining some information regarding what makes these mutants temperature sensitive or α-amanitin resistant. As shown by the dot-blot analysis in Fig. 2, both mutants were capable of replicating their DNA at the permissive and nonpermissive temperatures.

Late viral gene expression was analyzed by pulse-labeling infected cells at 8 h postinfection with [^{35}S]methionine. The qualitative and quantitative expression of viral proteins for both mutants was identical to that of the WT at the permissive and nonpermissive temperatures (Fig. 3). Early viral gene expression was also examined, with identical results (data not shown). To determine whether cleavage of the major core precursors P94 and P65 was taking place at the nonpermissive temperature, a pulse-chase experiment was

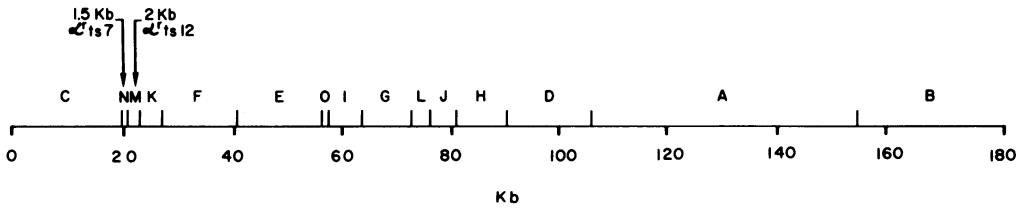

FIG. 1. *Hin*dIII restriction map of the whole VV genome identifying the fragments which rescue α^rts7 (*Hin*dIII-N) and α^rts12 (*Hin*dIII-M) to temperature insensitivity.

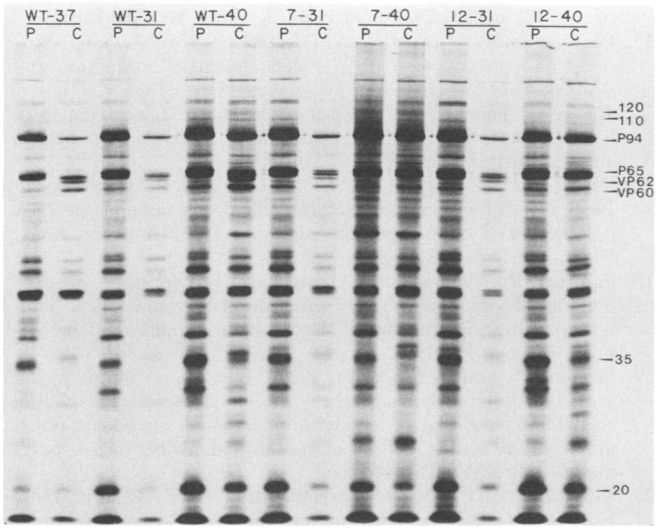

FIG. 3. Temperature-mediated effects of VV proteolytic cleavages. Cell monolayers were infected with α^rts7, α^rts12, or WT VV at a multiplicity of 25. At 8 h postinfection, the infected cells were pulse-labeled for 30 min with 5 μCi of [^{35}S]methionine per ml. Isotope-containing medium was then replaced with medium containing 10 mM unlabeled methionine, and infection was allowed to proceed for 8 h. Infected cell proteins were analyzed by polyacrylamide gel electrophoresis. The two major VV core proteins, VP62 and VP60, and their precursors, P94 and P65, are indicated. The numbers at the right indicate the molecular weights ($\times 10^3$) of marker proteins.

carried out late in infection. At the permissive temperature both mutants were capable of processing P94 and P65 into their products, VP62 and VP60, respectively (Fig. 3). At the nonpermissive temperature both mutants were unable to carry out the processing event.

DISCUSSION

In an effort to elucidate the nature of the host cell nuclear involvement in VV replication, we reported earlier (20) the isolation and characterization of a VV mutant (α-27) that is capable of replicating independently of the host cell nucleus. As it became obvious that further biochemical analysis of α-27 was unlikely to provide additional help in identifying the mechanism by which the mutant is capable of circumventing the need for the host cell nucleus, mapping the genomic location of the α-amanitin loci became imperative.

The first approach used, i.e., to marker rescue the replication of WT VV from α-amanitin-mediated inhibition by using cloned fragments of the α-27 genome, was hampered by the toxicity of α-amanitin, which made the use of single-step mapping procedures impossible. To overcome this obstacle we resorted to an alternative approach which involved the isolation of an α-amanitin resistant, temperature sensitive mutant in which both phenotypes were the result of one or two closely linked mutations. Screening of 60 mutants yielded 2 (α^rts7 and α^rts12) that met this criteria. Marker rescue experiments with the WT VV *Hin*dIII DNA fragments mapped the α^r locus within the 1.5-kb *Hin*dIII N fragment. These data suggest that a mutation observed earlier at the junction of the *Xho*I-O and *Xho*I-C fragments in α-27 (20) is not the site of the α^r locus.

The fact that no temperature-sensitive mutations in VV have previously been mapped to the left 28.5 kb of the genome (2, 4, 5), as well as the isolation of a viable deletion VV mutant (S variant) (14) that contains a 6.3-megadalton deletion in this same area, led researchers to speculate that this may represent a nonessential region of the genome. Recently Gillard et al. (6) revised this notion by showing that a host range mutant lacking 18 kb at the left-hand end of the genome, and which is unable to grow in human cell lines, can be restored to the WT phenotype by the addition of the *Eco*RI K fragment (5.0 kb), which encompasses most of the *Hin*dIII N fragment, the *Hin*dIII M fragment, and part of the *Hin*dIII K fragment. The fact that we were able to map two temperature-sensitive mutations as well as the α^r loci within the same region supports the idea that essential VV genes are present in this region of the genome.

Extensive transcriptional and translational analyses of this area of the VV genome by Belle Isle et al. (1) and by Morgan and Roberts (11) revealed that there are a number of early transcripts but no late transcripts encoded by the *Hin*dIII N fragment. One of these early transcripts, which is translated into a 20K polypeptide, seems to be fully encoded by the N fragment, whereas another early transcript, which is translated into a 55K polypeptide, is only partly encoded by this fragment. No function has been ascribed to either of the polypeptide products encoded partly or in full by the *Hin*dIII N fragment.

The data obtained thus far do not indicate in a definite manner what function is encoded by the α-amanitin resistance gene. A possible clue as to what an aspect of this function may be comes from the results of a pulse-chase experiment carried out in the presence of the drug which showed that WT VV is unable to process the major core precursors P94 and P65, whereas α-27 is capable of carrying out the processing event (20). This is reinforced by the finding that α^rts7, in which both phenotypes seem to be the result of a single mutation, is unable to carry out the processing event at the nonpermissive temperature (Fig. 3). By what mechanism and at what stage the processing event is stopped remain to be determined.

We are currently sequencing this region of the VV genome believed to contain the α^r gene to obtain the necessary information to allow identification of the encoded gene products.

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