Temperature-Sensitive Mutants of Frog Virus 3: Biochemical and Genetic Characterization

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Nineteen frog virus 3 temperature-sensitive mutants were isolated after mutagenesis with nitrosoguanidine and assayed for viral DNA, RNA, and protein synthesis, as well as assembly site formation at permissive (25°C) and nonpermissive (30°C) temperatures. In addition, mutants were characterized for complementation by both quantitative and qualitative assays. Based on the genetic and biochemical data, the 19 mutants, along with 9 mutants isolated earlier, were ordered into four phenotypic classes which define defects in virion morphogenesis (class I), late mRNA synthesis (class II), viral assembly site formation (class III), and viral DNA synthesis (class IV). In addition, we used two-factor crosses to order 11 mutants, comprising 7 complementation groups, onto a linkage map spanning 77 recombination units.

Frog virus 3 (FV3), the most studied member of the family *Iridoviridae*, is a large DNA-containing virus with a complex replication cycle (19, 40). Viral macromolecular synthesis is finely regulated and takes place in both the nucleus and cytoplasm of infected cells (21). Early mRNA synthesis and the formation of genome-length DNA progeny molecules (stage 1 of DNA synthesis) occur in the nucleus; whereas late viral message synthesis, viral protein synthesis, stage 2 DNA synthesis (formation of concatemers), and virion assembly take place in the cytoplasm, the latter in morphologically distinct viral assembly sites (16, 29, 35, 44).

Although there is considerable biochemical information on the synthesis of FV3 macromolecules in infected cells, there is a paucity of information at the genetic level. A limited number of FV3 temperature-sensitive (*ts*) mutants have been isolated (4, 17, 24, 32) and successfully utilized to analyze FV3 biogenesis (3, 17, 22-24, 34, 41). Continuation of these genetic and biochemical studies is important not only in elucidating the mechanism(s) of FV3 replication but also in understanding those aspects of FV3 biology that may serve as models for other systems. For example, learning how host RNA polymerase II transcribes the highly methylated FV3 genome (15, 45) will further our understanding of the regulatory effect of methylation on eucaryotic gene expression (7, 38). Toward these ends, we isolated and characterized 19 additional *ts* mutants and, along with 9 mutants isolated earlier, placed them into 19 complementation groups. Furthermore, we mapped 11 of these mutants by two-factor crosses and established a tentative genetic map. By combining genetic and biochemical data, we were able to order these mutants into four phenotypic classes which define defects in virion morphogenesis (class I), late gene activation (class II), assembly site formation (class III), and DNA synthesis (class IV).

MATERIALS AND METHODS

Virus and cells. A clonal isolate of wild-type (wt) FV3 was grown and assayed in fathead minnow (FHM) cells (32). Assays for viral macromolecular synthesis were performed with infected FHM cells; BHK cells were used for

immunofluorescent studies because these cells provided better specimens for microscopic examination than FHM cells (3).

Mutagenesis and isolation of *ts* mutants. FV3 *ts* mutants were isolated essentially as described by Naegle and Granoff (32). A cloned stock of FV3 was grown at 25°C in Eagle minimum essential medium containing 5% fetal calf serum and 1 µg of *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (MNNG; Sigma Chemical Co., St. Louis, Mo.). MNNG was chosen because it has been used successfully to obtain *ts* mutants in other viral systems (6, 9, 13). MNNG-grown virus was plated at 25°C; and well-separated plaques, isolated at random, were suspended in 1 ml of phosphate-buffered saline containing 5% fetal calf serum and tested for growth at 25°C (permissive temperature) and 30°C (nonpermissive temperature). Individual plaque isolates were used to infect duplicate monolayers of FHM cells in 96-well plates, and 3 days later the cultures were stained with crystal violet and scored for cytopathic effect. The absence of cytopathic effect at 30°C compared with 25°C was considered presumptive evidence for temperature sensitivity. Putative *ts* mutants were further tested by plaque assay at 25 and 30°C with the original plaque suspension as the inoculum. Mutants which formed plaques at 25°C, but not at 30°C, were recloned at least once and used to prepare virus stocks.

DNA synthesis. FHM cells grown on 35-mm-diameter dishes were infected with wt, heat-inactivated wt, or *ts* mutants at a multiplicity of 10 to 25 PFU per cell; and duplicate cultures were incubated at 25 and 30°C. Infected cells were labeled from 4 to 6 h after infection (when viral DNA synthesis was maximum [17]) with Eagle minimum essential medium containing 2% fetal calf serum and 5 µCi of [³H]methylthymidine (Schwarz/Mann, Orangeburg, N.Y.) per ml. At 6 h, the labeling medium was removed, the cells were disrupted in 10 mM Tris hydrochloride (pH 7.4)-10 mM KCl-1.5 mM MgCl₂ containing 1% Nonidet P-40 and 1% sodium dodecyl sulfate (SDS), and DNA was precipitated by the addition of three volumes of ice-cold ethanol. After the precipitate was washed 3 times with 5% trichloroacetic acid, the amount of [³H]methylthymidine incorporated into acid-insoluble material was determined by scintillation counting. Because heat-inactivated FV3 inhibits cellular DNA synthesis (17), the level of [³H]thymidine incorporated by cells

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infected with heat-inactivated wt FV3 was considered as background and subtracted from all values.

Protein synthesis. FHM cells, infected as described above, were pulse-labeled with MEM containing 25 μ Ci of [35 S]methionine (100 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml at the times and under the conditions indicated in the legend to Fig. 1 and 2. Radiolabeled cells were solubilized in 100 mM Tris hydrochloride (pH 6.8)–20% glycerol–2% SDS–2% β -mercaptoethanol, and viral polypeptides were analyzed by electrophoresis on SDS-polyacrylamide gels (25).

Viral RNA synthesis. Viral RNA, labeled with [3 H]uridine between 6 and 8 h after infection (a time when all viral RNA species are present [42, 43]), was isolated from 5×10^6 infected cells as described by Palmiter (33). Radiolabeled RNA was denatured in dimethyl sulfoxide-glyoxal (29), analyzed by electrophoresis on acid urea-agarose gels (28), and visualized by fluorography with En 3 Hance (New England Nuclear Corp., Boston, Mass.).

Visualization of viral assembly sites. BHK cells, grown on glass cover slips, were infected with wt *ts* mutants at 50 to 100 PFU per cell, and replicate cultures were incubated at permissive and nonpermissive temperatures for 16 h. High multiplicities of infection were used to ensure infection of all cells. Assembly sites were visualized by indirect immunofluorescence with mouse anti-FV3 serum and fluorescein isothiocyanate-conjugated goat anti-mouse serum as described previously (3). Alternatively, assembly sites were visualized by Feulgen staining (3) or electron microscopy (21).

Complementation and recombination. Quantitative complementation and recombination tests were performed as described by Naegle and Granoff (32), except that an input multiplicity of 7 PFU of each virus per cell was used. For quantitative complementation, FHM cells, infected either mixed or singly with *ts* mutants, were incubated for 1 h at 30°C and then washed three times to remove unabsorbed virus. Prewarmed growth medium was added, and incubation continued for 24 h at 30°C, at which time the virus was harvested. The total virus yield was determined by plaque assay at 25°C, and the complementation index (CI) was calculated by dividing the yield produced from mixed infection by the sum of the yields produced by the two mutants grown separately. All values for the CI were rounded to the nearest whole number, and values greater than 2 were considered indicative of complementation (13). For recombination, mixed infected cultures were incubated at 25°C for 24 h, and the virus yield was assayed at both 25 and 30°C to determine, respectively, total yield and the yield of wt recombinants. The recombination frequency was calculated as follows: (mixed yield of A $\times$ B, assayed at 30°C/mixed yield of A $\times$ B, assayed at 25°C) $\times$ 2 $\times$ 100. Because our mutants were not leaky and did not revert at high frequencies, no correction for these events was necessary when the recombination frequency was determined. Qualitative complementation tests were performed as described by Condit and Motyczka (6) with an input multiplicity of 0.01 PFU per cell.

RESULTS

Isolation and characterization of FV3 *ts* mutants. To facilitate the isolation of *ts* mutants, wt FV3 was grown in medium containing 1 μ g of MNNG per ml, a compound which reduced the virus yield by 60% and, as shown below, induced mutations at a high frequency. Subsequently, FHM monolayers grown on 60-mm-diameter dishes were inocu-

TABLE 1. Properties of FV3 *ts* mutants

Virus	EOP at 30°C/25°C ($\times 10^{-6}$) ^a	Leakiness ^b (%)	DNA synthesis (% $\pm$ SD) ^c	Protein synthesis ^d	Assembly sites ^e
wt	570,000	234	161 $\pm$ 78	wt	+
<i>ts1</i>	4	7	82 $\pm$ 9	wt	+
<i>ts2</i>	7	1	108 $\pm$ 21	wt	+
<i>ts3</i>	5	6	131 $\pm$ 9	wt	+
<i>ts5</i>	9	<1	7 $\pm$ 6	Early	–
<i>ts7</i>	10	1	210 $\pm$ 128	wt	+
<i>ts13</i>	30	<1	5 $\pm$ 5	Early	–
<i>ts16</i>	4	3	120 $\pm$ 2	wt	+
<i>ts17</i>	3	<1	90 $\pm$ 18	wt	+
<i>ts19</i>	4	<1	191 $\pm$ 52	wt	+
<i>ts20</i>	5	1	16 $\pm$ 11	Early	–
<i>ts21</i>	2	<1	87 $\pm$ 50	wt	+
<i>ts22</i>	6	2	116 $\pm$ 4	wt	+
<i>ts12460</i>	2	<1	108 $\pm$ 36	Early	$\pm$
<i>ts12738</i>	1	2	51 $\pm$ 31	Early	$\pm$
<i>ts12759</i>	1	<1	270 $\pm$ 160	wt	+
<i>ts12783</i>	2	3	197 $\pm$ 112	wt	+
<i>ts12810</i>	3	1	253 $\pm$ 99	wt	+
<i>ts12814</i>	1	1	59 $\pm$ 22	wt	+
<i>ts12818</i>	3	2	72 $\pm$ 19	Early	–

^a EOP, Efficiency of plating. Data are the averages from two to four experiments.

^b Leakiness = (virus yield at 30°C/virus yield at 25°C) $\times$ 100. FHM cells were infected with 5 to 10 PFU per cell and incubated at 25 or 30°C for 24 h, and the titer of the virus yield was determined at 25°C. Data are the averages from two to four experiments.

^c Percent DNA synthesis = (value at 30°C/value at 25°C) $\times$ 100. Viral DNA synthesis was measured as described in the text. Data are the averages from two to four experiments.

^d The ability of *ts* mutants to express viral proteins was determined as described in the text. wt, normal expression of early and late viral proteins; early, predominantly early proteins, with few if any late proteins, were synthesized at 30°C.

^e Assembly site formation was determined by Feulgen staining, indirect immunofluorescence, and electron microscopy. +, presence of assembly sites; –, absence of assembly sites; $\pm$ assembly sites are smaller in size and less numerous at 30°C than at 25°C.

lated with 50 to 100 PFU of MNNG-treated virus and incubated at 25°C in medium containing agar. Because presumptive FV3 *ts* mutants cannot be identified by plaque enlargement, well-isolated, randomly selected plaques were harvested and screened for growth at 25 and 30°C on replicate monolayers of FHM cells grown on 96-well plates. Titers of putative mutants were determined at 25 and 30°C; and isolates which yielded plaques at the permissive, but not at the nonpermissive, temperature were plaque purified and used to prepare small stocks. To ensure that *ts* mutants were genetically stable and suitable for biochemical analysis, only mutants that had low frequencies of reversion (efficiency of plating at 30°C/efficiency of plating at 25°C, less than 10^{-5}) and low levels of leakiness (yield at 30°C/yield at 25°C, less than 10^{-1}) were retained (Table 1). Mutants that met these criteria were detected at a frequency of nearly 2% (in one experiment, 12 of 729 isolates yielded stable, nonleaky mutants) and were further characterized as described below.

Viral DNA synthesis. To identify *ts* mutants defective in viral DNA synthesis, we measured the incorporation of [3 H]thymidine in *ts* mutant-infected cells at 25 and 30°C. At the nonpermissive temperature, 3 of 19 mutants (*ts5*, *ts13*, and *ts20*) synthesized markedly reduced amounts of DNA (Table 1) which, by restriction enzyme analysis, was shown to be virus rather than host specific (data not shown). Subsequent complementation analysis (Table 2) indicated

TABLE 2. Quantitative complementation analysis between FV3 *ts* mutants^a

<i>ts</i> mutant	CI. for the following <i>ts</i> mutants:													
	1	2	3	5	7	13	16	17	19	20	21	22	6642	12488
1		14	18	18	9	22	27	22	ND ^b	73	63	41	20	16
2			<u>1</u>	110	63	145	38	34	45	104	<u>2</u>	45	37	31
3				57	611	50	3	6	4	56	<u>1</u>	9	54	29
5					96	324	22	8	5	166	233	18	10	1
7						431	12	3	32	363	14	<u>1</u>	ND	ND
13							29	15	78	<u>1</u>	46	60	<u>1</u>	57
16								147	645	656	27	36	ND	ND
17									84	236	19	31	ND	ND
19										358	3	46	ND	ND
20											86	106	<u>1</u>	50
21												13	ND	ND
22													ND	ND
6642														9
12488														

^a Complementation was performed as indicated in the text. The values given are the means of at least two experiments. CI ≤ 2 indicate that mutants are defective in the same gene and are underlined to identify noncomplementing pairs of mutants.

^b ND, Not done.

that *ts* 13 and 20 were in the same complementation group as *ts* 6642, a previously described mutant that is defective in the first stage (DNA₋₁) of viral DNA synthesis (24), whereas *ts* 5 was in the same group as *ts* 12488, a mutant defective in the second stage (DNA₋₂) of FV3 DNA synthesis (17). Although DNA₋₁ mutants (*ts* 13, *ts* 20, and *ts* 6642) are defective in their ability to initiate viral DNA synthesis (24), it is not known whether the defect in DNA₋₂ mutants (*ts* 5 and *ts* 12488) results from an inability to synthesize concatemeric DNA or from a block in the transport of newly synthesized DNA from the nucleus to the cytoplasm (17). Of the remaining 16 mutants, 10 synthesized wt levels of DNA at 30°C (note that approximately twice as much DNA was synthesized by wt virus and some *ts* mutants at 30°C than at 25°C), whereas six mutants (*ts* 1, *ts* 17, *ts* 21, *ts* 12738, *ts* 12814, and *ts* 12818) produced intermediate yields (DNA[±]). It is not known whether the DNA[±] phenotype reflects a partial defect in the viral polymerase (4) or in another protein required for DNA synthesis or whether it is a pleiotropic manifestation of a defect in an unrelated gene.

Viral protein synthesis. In FV3-infected cells, viral protein synthesis occurs in three distinct virus-controlled temporal phases in which immediate-early, delayed-early, and late proteins are expressed sequentially (12, 44). To determine whether *ts* mutants were defective in the synthesis of one or more classes of viral protein, we examined protein synthesis at permissive and nonpermissive temperatures by pulse-labeling mutant-infected cells from 6 to 8 h after infection with [³⁵S]methionine and by analyzing radiolabeled proteins on one-dimensional SDS-polyacrylamide gels. We found that our mutants could be divided into two groups. Thirteen mutants synthesized essentially wt levels of early (here we consider the immediate-early and delayed-early classes together) and late proteins at the nonpermissive temperature, whereas six mutants were markedly defective in late protein synthesis (Table 1). Representative patterns of protein synthesis from wt- and mutant-infected cells are shown in Fig. 1 and 2. *ts* 16 synthesized an essentially wt pattern of viral proteins at both 25 and 30°C; whereas in cells infected with *ts* 12738, a mutant defective in late gene expression, translation at the nonpermissive temperature was confined mainly to the synthesis of early [108,000 daltons [108K], 45K, 36K, and 18K] virus-specific proteins (12, 42) (Fig. 1). Although the synthesis (and often overproduction) of early viral pro-

teins was characteristically seen in cells infected with mutants defective in late gene expression, this block was not absolute, and low levels of some late proteins were sometimes observed. The low level of late gene expression was probably due to leakiness and was multiplicity dependent (24). Kinetic studies (Fig. 2) confirmed the results of our rapid protein screens and showed that mutants able to synthesize wt levels of both early and late viral proteins (e.g., *ts* 16) made the characteristic transition from early to late protein synthesis at both permissive and nonpermissive temperatures, whereas mutants defective in late gene expression (e.g., *ts* 12738) remained locked in the early pattern of protein synthesis even at late times after infection. Lastly, we observed that all *ts* mutants inhibited host cell protein synthesis at both 25 and 30°C (compare *ts* 16 and *ts* 12738 in Fig. 1 and 2 with mock-infected cells), indicating that no mutant was defective in the virus-induced shutoff of host cell translation.

FV3 mRNA synthesis. Because the inability to synthesize wt levels of late proteins could result either from a specific block in the translation of late messages (36) or from a defect in the synthesis of late mRNAs themselves, we examined mRNA synthesis in FHM cells infected with mutants defective in late protein synthesis. Besides the six mutants described above, we included in this study six additional mutants (*ts* 150, *ts* 2436, *ts* 3674, *ts* 6642, *ts* 9467, and *ts* 12488) that were isolated previously and shown to be defective in late protein synthesis (17, 22, 24, 41). FV3-specific mRNA was labeled by the addition of [³H]uridine to infected cells between 6 and 8 h after infection, a time when all virus-specific messages are present and host cell RNA synthesis is inhibited (44). Radiolabeled RNA was isolated, denatured with dimethyl sulfoxide-glyoxal, and analyzed by electrophoresis on acid urea-agarose gels (Fig. 3). As an internal control, viral protein synthesis was monitored in replicate cultures by electrophoresis of [³⁵S]methionine-labeled proteins on 9% SDS-polyacrylamide gels (data not shown). Analysis of the RNA products synthesized in mutant-infected cells at 25 and 30°C indicated that mutants defective in the expression of late proteins were also defective in the synthesis of late mRNA (Fig. 3). Note that inhibition of DNA synthesis by phosphonoacetate (4, 11) also blocks the synthesis of late mRNAs and mimics the phenotype of mutants defective in late gene expression (Fig. 3, lane 3).

FIG. 1. Protein synthesis by wt FV3 and representative *ts* mutants in FHM cells under permissive and restrictive growth conditions. FHM cells were mock infected (M; lane 2), or infected with wt virus or *ts* mutants at a multiplicity of infection of 10 to 25 PFU per cell. Replicate cultures of *ts* mutant-infected cells were incubated at 25 and 30°C whereas wt-infected cells were incubated at 25°C. Replicate cultures of wt-infected cells were maintained in drug-free medium (lane 1) or in medium containing 50 μ g of cycloheximide (CH; lane 4) per ml or 200 μ g of phosphonoacetate (PAA; lane 3) per ml. Addition of these drugs resulted in, respectively, the selective synthesis of immediate-early (44) and both immediate-early and early proteins (4, 12). Early and late viral proteins were radiolabeled from 6 to 8 h after infection in medium containing 25 μ Ci of [35 S]methionine per ml, separated by electrophoresis on 12% SDS-polyacrylamide gels, and visualized by autoradiography. Immediate-early proteins (i.e., cycloheximide-treated cultures) were labeled, following removal of the cycloheximide, from 4 to 6 h after infection in [35 S]methionine-containing medium plus actinomycin D (10 μ g/ml). Characteristic early (○) and late (no symbol) virus-specific proteins are identified by their molecular mass.

When late mRNAs were detected (presumably a reflection of leakiness), a corresponding increase in the amount of late protein synthesis was also seen. In no case did we observe a wt pattern of late mRNA synthesis in the absence of late protein synthesis. This correlation between levels of late mRNA and late protein in mutant-infected cells at 30°C suggests that the primary *ts* defect in mutants defective in late gene expression is in the turn-on (activation) of late gene transcription and not in late message translation.

Viral assembly site formation and virion assembly. The formation of FV3 virions is a complex process that occurs in morphologically defined regions of the cytoplasm, termed assembly sites (3, 40). Because assembly site formation and virion morphogenesis are virus-directed functions (3), we analyzed our panel of *ts* mutants for defects in these properties. At the nonpermissive temperature the majority of mutants (13 of 19) formed large wt assembly sites which were readily visualized by Feulgen staining, indirect immunofluorescence, and electron microscopy (Fig. 4; unpublished data). In Fig. 4 are shown cells infected by two representative *ts* mutants in which assembly sites were visualized by immunofluorescence. Note that viral assembly sites can be clearly seen in *ts*19-infected cells at both 25 and 30°C, whereas assembly sites were detected in *ts*5-infected

cells only at 25°C. We observed that all mutants that synthesized wt levels of viral macromolecules at the nonpermissive temperature were also competent to synthesize assembly sites at 30°C. However, in accord with previous results (34), electron microscopic observation of representative mutants indicated that none of these formed recognizable previrion or virionlike particles at 30°C (data not shown). In addition, four mutants (three defective in DNA synthesis and one DNA⁺) failed to form any assembly sites at 30°C, while two others that were defective in late viral gene expression (*ts*12460 and *ts*12738) formed assembly sites that were smaller and less numerous than those formed

FIG. 2. Kinetics of protein synthesis by FV3 *ts* mutants in FHM cells at permissive and nonpermissive temperatures. FHM cells were infected with representative *ts* mutants at a multiplicity of 25 PFU per cell and labeled with 25 μ Ci of [35 S]methionine per ml for 1-h intervals beginning at the times indicated at the bottom of each gel. Samples, representing equal numbers of infected cells, were electrophoresed on 5 to 15% SDS-polyacrylamide gels, and viral proteins were visualized by autoradiography. Radiolabeled uninfected (UN) FHM cells and purified virions (V) were included as markers for host proteins and virion structural proteins (VP). Virus-specific proteins are identified by their molecular mass (in kilodaltons).

FIG. 3. Viral mRNA synthesis in FHM cells infected with wt virus and *ts* mutants. Replicate cultures of FHM cells were infected with representative *ts* mutants at 10 PFU per cell and incubated at 25 and 30°C or with wt FV3 at the same multiplicity of infection and incubated in the presence or absence of 200 µg of phosphonoacetate (PAA) per ml. Infected cells were incubated in medium containing 50 µCi of [³H]uridine per ml from 6 to 8 h after infection, and viral RNA, extracted with SDS-phenol-chloroform, was isolated following precipitation with ethanol and LiCl (33). Radiolabeled RNA was subsequently denatured with dimethyl sulfoxide-glyoxal (30), and equal numbers of counts per minute were analyzed by electrophoresis on 1.5% acid urea-agarose gels (28). Characteristic early and late messages are indicated.

at 25°C (Table 1). The variable response seen with the last two mutants may be due to leakiness and is probably multiplicity dependent.

Quantitative complementation. To determine whether mutants with similar phenotypes were defective in the same genes, mutants were ordered into complementation groups by a quantitative complementation test. Initially, we assayed 14 mutants (12 newly isolated ones and 2 previously described DNA⁻ mutants) and observed CIs that ranged from 1 to greater than 600. Assuming that a CI greater than 2 indicated mutants defective in different genes (13), 14 mutants were ordered into eight complementation groups (Table 2). Although the quantitative assay generally gave unambiguous results, several mutants (*ts*3, *ts*5, *ts*17, and *ts*19) behaved anomalously in that they showed reduced (but positive) levels of complementation (i.e., CIs ranged from 2 to 10) in a limited number of crosses. It is not known whether these low values are due to interallelic complementation or leakiness. However, with the exception of *ts*3, the latter possibility is unlikely because *ts*5, *ts*17, and *ts*19 synthesized 100-fold less virus at 30°C than at 25°C (Table 1). As discussed below, confirmation of the assignment of these

four mutants to different complementation groups (as originally suggested by their CIs) involved comparison of phenotypic differences and complementation-recombination data. Surprisingly, the three DNA⁻ mutants isolated in this study (*ts*5, *ts*13, and *ts*20) were found to be members of two previously described complementation groups, defined by *ts*6642 and *ts*12488 (17, 24). Furthermore, the presence of *ts*20 in the same complementation group as *ts*6642 confirmed its designation as a DNA⁻ mutant, even though there was some DNA synthesis at 30°C (Table 1).

Recombination. Because it is likely that mutants defective in the same gene recombine poorly, whereas mutants in different genes recombine (depending on the distance separating them) with high efficiency, we measured recombination frequencies in pairwise crosses involving 11 of the 14 *ts* mutants tested for complementation. Our aim here was twofold. First, we wanted to determine whether CIs correlated with recombination frequencies and therefore whether they could be used to confirm the assignment of mutants to complementation groups. Second, we wanted to construct a genetic map for FV3. We observed, in agreement with our own earlier observations (32) and those of others (2, 6, 9, 13),

FIG. 4. FV3 assembly site formation by representative *ts* mutants. BHK cells grown on glass cover slips were infected with FV3 *ts* mutants at 100 PFU per cell, and replicate cultures were incubated at 25 and 30°C. Sixteen hours later, the cells were fixed and processed for immunofluorescence with mouse anti-FV3 and fluorescein isothiocyanate-conjugated rabbit anti-mouse serum as the first and second antibodies, respectively (3). Assembly sites are indicated by arrows.

that recombination frequencies between mutants in different genes were generally high and showed considerable variation between experiments (Table 3). Attempts at lowering this variation by harvesting virus at earlier times after infection (e.g., at 8 h rather than 24 h) did not alter the results. As postulated, mutants in the same complementation group (Table 3) showed much lower recombination frequencies than mutants in different complementation groups with one exception (*ts3* × *ts21*). This observation suggests that ordering mutants into complementation groups may be facilitated by comparing data from phenotypic (Table 1), complementation (Table 2), and recombination (Table 3) assays. CIs and recombination frequencies from representative crosses involving mutants (*ts3*, *ts5*, *ts17*, and *ts19*), the assignment of which to complementation groups was ambiguous, were compared, and the results are shown in Table 4. We conclude that *ts3*, *ts5*, *ts17*, and *ts19* could be assigned to different complementation groups based on one or more of the following criteria: (i) high frequencies of recombination in crosses that gave CIs of 10 or less, (ii) differences in the level of viral DNA synthesis at 30°C, and (iii) high levels of complementation in crosses involving a second mutant in the same complementation group. For example, although *ts3* × *ts17* yielded a CI of 6, *ts2*, another mutant in the same

complementation group as *ts3*, complemented *ts17* efficiently (i.e., CI = 34). These results confirm the assignment of 11 newly isolated *ts* mutants to eight distinct complementation groups and indicate the appropriateness of using a CI of greater than 2 as the cutoff for complementation.

Using the recombination frequencies obtained in two-factor crosses (Table 3), we were able to establish a linkage map for 11 *ts* mutants. Because recombination frequencies greater than 20% are not additive (13, 27) and many of our values were in that range, a genetic map was constructed by determining gene order based on several pairwise crosses involving six to eight mutants and by using the consensus order from those studies to produce a composite map. The resultant map (Fig. 5) spans 77 recombination units and, although tentative, will serve as the basis for continued genetic mapping of FV3 by marker rescue. The cloning of greater than 95% of the FV3 genome (R. Goorha and V. G. Chinchar, unpublished data) provides an abundant source for purified restriction fragments and will facilitate marker rescue. Interestingly, despite similar problems with additivity, the gene order of two independently derived genetic maps of vaccinia virus have been confirmed by marker rescue (8, 13).

Qualitative complementation analysis. Because analysis of

TABLE 3. Recombination frequencies for pairs of *ts* mutants

<i>ts</i> mutant	Recombination frequencies $\pm$ SD for the following <i>ts</i> mutants										
	16	2	3	21	19	17	5	7	22	13	20
16		26 $\pm$ 10	35 $\pm$ 12	23 $\pm$ 1	21 $\pm$ 2	26 $\pm$ 4	17 $\pm$ 1	20 $\pm$ 6	18 $\pm$ ND ^b	36 $\pm$ 2	51 $\pm$ 16
2			2 $\pm$ 2	2 $\pm$ 1	11 $\pm$ 3	10 $\pm$ 4	13 $\pm$ 5	11 $\pm$ 3	12 $\pm$ 5	25 $\pm$ 8	18 $\pm$ 4
3				7 $\pm$ 2	17 $\pm$ 5	5 $\pm$ 2	19 $\pm$ 4	12 $\pm$ 7	16 $\pm$ 8	31 $\pm$ 18	25 $\pm$ 3
21					20 $\pm$ 7	21 $\pm$ 9	8 $\pm$ 1	9 $\pm$ 4	18 $\pm$ 16	15 $\pm$ 3	19 $\pm$ 5
19						11 $\pm$ 4	12 $\pm$ 4	10 $\pm$ 4	22 $\pm$ 2	25 $\pm$ 4	20 $\pm$ 8
17							4 $\pm$ 2	6 $\pm$ 4	6 $\pm$ ND	18 $\pm$ 3	25 $\pm$ 5
5								9 $\pm$ 7	11 $\pm$ 4	19 $\pm$ 5	13 $\pm$ 5
7									2 $\pm$ 2	12 $\pm$ 2	16 $\pm$ 3
22										18 $\pm$ 1	32 $\pm$ 16
13											1 $\pm$ 1

^a Recombination frequencies were calculated as described in the text. Each cross was done at least twice, and the recombination frequency and standard deviation are shown. Mutants in the same complementation group are underlined.

^b ND, Not done.

large numbers of mutants by the quantitative complementation assay is time consuming, we employed a qualitative assay to screen the remaining mutants. Similar assays have been used successfully to map mutants of bacteriophage T4, herpes simplex virus, and vaccinia virus (5, 6, 10). In the qualitative assay, FHM cells were infected at a multiplicity of 0.01 PFU per cell, and cells coinfecting with complementing mutants served as foci for plaque formation. Although complementation is the predominant genetic event initially, recombination becomes important later because plaque formation requires multiple rounds of replication and wt recombinants arising in early rounds are amplified and contribute significantly to the final yield (6, 13). Although the occurrence of recombination may seem, at first, to negate the utility of this assay, this is not so. Because mutants in different complementation groups recombine freely, whereas those in the same group do not, the occurrence of recombinants in the qualitative test should enhance plaque formation by mutants defective in different genes, while mutants in the same gene (because they do not complement and recombine poorly) should not give rise to false positive (6, 13).

Table 5 shows the results of pairwise crosses involving 27 *ts* mutants (19 new plus 8 previous isolates). There was good agreement between the qualitative complementation assay (Table 5) and the more laborious quantitative assay (Table 2), except for one cross (*ts5* $\times$ *ts6642*). This discrepancy is likely due to the fact that *ts5* and *ts6642* are both defective in DNA synthesis and, as suggested by Ensinger (13), some DNA⁻ mutants, even with defects in different genes, recombine so poorly at nonpermissive temperatures that they fail to produce plaques at 30°C.

In mutants not tested previously by quantitative assay, nine noncomplementing crosses were detected (Table 5). Only one of these (*ts1* $\times$ *ts1912*) has been confirmed as a true negative by quantitative assay, whereas eight others were probably false negatives as indicated by subsequent quantitative assay (i.e., *ts5* $\times$ *ts12738*, *ts3674* $\times$ *ts12738*, *ts6642* $\times$ *ts12738*, *ts12738* $\times$ *ts12818*) or by dissimilarities in their biochemical phenotypes (*ts2* $\times$ *ts2436*, *ts5* $\times$ *ts3674*, *ts17* $\times$ *ts6642*, *ts6642* $\times$ *ts12818*). Note that in all cases involving false negatives, one or both members of the cross were mutants defective in either DNA synthesis or the synthesis of late viral proteins. This observation suggests that, at least in some crosses, plaque formation in the qualitative assay (i.e., complementation) requires wt levels of gene expression. These results indicate that rapid screening of *ts* mu-

tants by a qualitative assay is just as sensitive an indicator of complementation as the more laborious quantitative assay. Thus, we were able to order efficiently 27 FV3 *ts* mutants into 19 complementation groups (Table 6).

DISCUSSION

FV3 complementation groups and phenotypic classes. Based on the biochemical and genetic analyses reported here and in several previous reports (3, 17, 22, 24, 32, 34, 41), we

TABLE 4. Assignment of *ts* mutants to complementation groups based on CIs, recombination frequencies, and phenotype

Cross	CI ^a	Recombination frequency ^b	Complementation
<i>ts2</i> $\times$ <i>ts3</i>	1	2	—
<i>ts2</i> $\times$ <i>ts21</i>	2	2	—
<i>ts2</i> $\times$ <i>ts19</i>	45	11	+
<i>ts2</i> $\times$ <i>ts17</i>	34	10	+
<i>ts2</i> $\times$ <i>ts5</i>	110	13	+
<i>ts3</i> $\times$ <i>ts16</i>	3	35	+
<i>ts3</i> $\times$ <i>ts21</i>	1	7	—
<i>ts3</i> $\times$ <i>ts19</i>	4	17	+
<i>ts3</i> $\times$ <i>ts17</i>	6	5	+ ^c
<i>ts3</i> $\times$ <i>ts5</i>	57	19	+
<i>ts3</i> $\times$ <i>ts22</i>	9	16	+
<i>ts21</i> $\times$ <i>ts19</i>	3	20	+
<i>ts21</i> $\times$ <i>ts17</i>	19	21	+
<i>ts21</i> $\times$ <i>ts5</i>	233	8	+
<i>ts19</i> $\times$ <i>ts17</i>	84	11	+
<i>ts19</i> $\times$ <i>ts5</i>	5	12	+
<i>ts17</i> $\times$ <i>ts5</i>	8	4	+ ^d
<i>ts17</i> $\times$ <i>ts22</i>	31	6	+
<i>ts7</i> $\times$ <i>ts17</i>	3	6	+ ^c
<i>ts7</i> $\times$ <i>ts22</i>	1	2	—
<i>ts13</i> $\times$ <i>ts20</i>	1	1	—

^a From Table 2.

^b From Table 3.

^c These mutants were classified as members of separate complementation groups based on a CI $>$ 2 in the quantitative assay and formation of plaques in the qualitative assay (Table 5).

^d Assignment of *ts5* and *ts17* to separate complementation groups is based on a CI of $>$ 2 and on different phenotypes (*ts5* is DNA⁻, *ts17* is DNA⁺).

FIG. 5. Linkage map of FV3 *ts* mutants. FHM cells were infected singly or with pairs of *ts* mutants (7 PFU of each per cell) and incubated at 25°C, and virus yields were assayed at 30°C. Recombination frequencies were determined as described previously (32). When there was more than one mutant in a complementation group, at least two were used in crosses with other mutants. The distances given are the mean recombinational frequencies (± 1 standard deviation) obtained from two or more crosses. Crosses involving mutants in the same complementation group are indicated by frequencies contained within brackets.

ordered 28 FV3 *ts* mutants into four phenotypic classes (Table 6). The four classes, defining defects in virion morphogenesis (class I), late gene activation (class II), assembly site formation (class III), and DNA synthesis (class IV), are discussed below.

Mutants defective in virion formation (class I). Class I mutants, comprising 12 complementation groups, synthesized wt levels of viral macromolecules and formed viral assembly sites but failed to assemble infectious particles at the nonpermissive temperature. The large number of complementation groups (i.e., genes) defective primarily in virion morphogenesis suggests that virion formation is a complex process requiring multiple proteins, mutation in any of which will prevent virion formation. Compatible with this view is the observation that class I mutants failed to form virion or previrion particles at the nonpermissive temperature (34; B. Tarnowski and G. Murti, unpublished data). The large number of complementation groups (12 of 19) involved in virion morphogenesis is consistent with the large number of viral structural proteins (22 proteins [12]), the complex

structure of iridoviruses (1, 19), and the enzymatic requirements for packaging headful amounts of circularly permuted and terminally redundant genomes (20). Furthermore, morphogenesis-negative mutants may be detected more often than other *ts* mutants because it is unlikely that host functions can compensate for defects in virion morphogenesis, whereas other mutants (e.g., DNA⁻ mutants) may be complemented by functionally similar host enzymes. We anticipate that these mutants will be useful in examining the mechanisms of virion morphogenesis, including the packaging of virion DNA.

Mutants defective in late gene expression (class II). Class II mutants are defective in their ability to synthesize late messages and proteins at 30°C but synthesize early proteins, viral DNA, and form virus assembly sites under nonpermissive conditions. Based on the observed correlation between levels of late mRNA and protein, we feel that the primary defect in class II mutants is in the activation (turn-on) of late gene transcription. This defect is in contrast to those seen in class III and class IV mutants, in which (as we suggest

TABLE 5. Qualitative complementation analysis between FV3 *ts* mutants

<i>ts</i> Mutant	Complementation analysis results for the following <i>ts</i> mutants ^a :																											
	1	2	3	5	7	13	16	17	19	20	21	22	150	1912	2436	2823	3589	3674	6642	12460	12488	12738	12759	12783	12810	12814	12818	
1		ND ^b	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2			−	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
3				+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5					+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	−	+	+	+	+	+	+
7						+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13							+	+	+	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
16								+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19										+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20											+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21												+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22													+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
150														+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1912															+	+	+	+	+	+	+	+	+	+	+	+	+	+
2436																+	+	+	+	+	+	+	+	+	+	+	+	+
2823																	+	+	+	+	+	+	+	+	+	+	+	+
3589																		+	+	+	+	+	+	+	+	+	+	+
3674																			+	+	+	−	+	+	+	+	+	+
6642																				+	+	−	+	+	+	+	+	−
12460																					+	+	+	+	+	+	+	+
12488																						+	+	+	+	+	+	+
12738																							+	+	+	+	+	+
12759																								+	+	+	+	−
12783																									+	+	+	+
12810																										+	+	+
12814																										+	+	+
12818																											+	+

^a FHM monolayers grown on 35-mm-diameter dishes were infected singly or with different *ts* mutants at 0.01 PFU per cell, incubated at 30°C for 4 days, and then stained with crystal violet (5). The presence of viral plaques on monolayers infected with two different mutants, in which no plaques appeared on the corresponding monolayers infected singly, indicated complementation (+). The absence of plaques on mixed infected monolayers suggested that complementation did not occur (-).

^b ND, Not done.

below) the inability to synthesize late proteins is due either to an inability to form virus assembly sites (class III) or to a defect in DNA synthesis (class IV).

Although results of biochemical studies (23, 36) also suggest the existence of a late gene activator, no information on its identity is available. Goorha et al. (23) found that at least two gene products are required for wt levels of late transcription; one product serves to activate late transcription and the second controls the abundance of late messengers. However, the identity of these regulatory proteins was not determined. It is surprising that four different class II complementation groups show defects in late gene activation because it is unlikely that four different gene products are directly involved in this event. We speculate that only one mutant protein may be directly involved in the turn-on of late gene transcription, whereas the other three gene products may act in a pleiotropic manner to produce the class II phenotype. Alternatively, the ambiguity in interpreting complementation studies involving mutants defective in late gene expression (discussed above) may mean that the ordering of five class II mutants into four distinct complementation groups is incorrect and that there actually are fewer class II complementation groups. Continued study of these mutants may identify key regulatory proteins that control late gene transcription.

Mutants defective in assembly site formation (class III). These mutants, like those of class II, are defective in late viral functions, but in addition, they fail to form assembly sites at the nonpermissive temperatures (Table 6). In an earlier study utilizing *ts*9467 (3), we showed that only early

proteins and viral DNA are required for assembly site formation and that at least one early protein required for assembly site formation was synthesized at 30°C, although it was functionally inactive. The absence of both assembly

TABLE 6. Phenotypic classes of FV3 *ts* mutants

Class	Complementation group	<i>ts</i> Mutant	Early protein	Late protein	DNA synthesis	Assembly sites
I	A	1, 1912	+	+	+	+
	B	2, 3, 21	+	+	+	+
	C	7, 22	+	+	+	+
	D	16	+	+	+	+
	E	17	+	+	+	+
	F	19	+	+	+	+
	G	2823	+	+	+	+
	H	3589	+	+	+	+
	I	12759	+	+	+	+
	J	12783	+	+	+	+
	K	12810	+	+	+	+
	L	12814	+	+	+	+
	M	150, 3674	+	-	+	±
II	N	2436	+	-	+	±
	O	12460	+	-	+	±
	P	12738	+	-	+	±
III	Q	9467, 12818	+	-	+	-
IV	R	13, 20, 6642	+	-	- ^a	-
	S	5, 12488	+	-	- ^b	-

^a Defective in the first stage of FV3 DNA synthesis.

^b Defective in the second stage of FV3 DNA synthesis.

sites and late gene expression in *ts9467*-infected cells coupled with genetic evidence that *ts9467* is not a double mutant suggest that late message synthesis requires functional viral assembly sites. Preliminary data consistent with this hypothesis show that late message synthesis and assembly site formation reappear simultaneously when *ts9467*-infected cells are shifted from nonpermissive to permissive temperatures (V. G. Chinchar, unpublished data). Class III mutants will be useful in further defining the requirements for assembly site formation and for elucidating their function.

Mutants defective in viral DNA synthesis (class IV). Phenotypic class IV comprises two complementation groups that are both defective in viral DNA synthesis, and mutants from each of these complementation groups have been used to elucidate the two-stage mechanism of FV3 DNA replication (17, 24). Mutants in group R (*ts13*, *ts20*, and *ts6642*) are defective in the first stage of viral DNA synthesis, the formation of genome-length daughter molecules in the nucleus (24), whereas group S mutants (*ts5* and *ts12488*) are defective in the second stage of DNA replication. As discussed above, it is not known whether these mutants are defective in the viral polymerase itself or in another protein required for viral DNA replication. Moreover, it is not clear whether the inability of group S mutants to synthesize concatemers is due to a block in the transport of viral DNA from the nucleus to the cytoplasm or to a defect in the process of concatemer formation (24).

The small number of complementation groups (2 of 19) defective in DNA synthesis is surprising in light of the complex scheme of FV3 DNA replication (16, 20). However, a similarly low number of complementation groups defective in DNA synthesis has been found in vaccinia virus (13). Perhaps fewer genes than we suspect are involved in DNA synthesis, or the gene products involved have such stringent conformational requirements that any change in their active sites abolishes activity. Alternatively, mutation in some functions required for viral DNA synthesis may lead to a decrease in, but not elimination of, replication, whereas other *ts* defects may be masked by complementation involving functionally similar cellular enzymes (e.g., host cell thymidine kinase, exonuclease, ligase). The observation that FV3 DNA polymerase is inhibited by aphidicolin (4), an inhibitor of eucaryotic DNA polymerase α , suggests that despite the unique structure of FV3 DNA, enough similarity may exist between FV3 and host cell DNA replication to permit the substitution of some host replicative enzymes for defective viral ones.

All mutants defective in DNA synthesis were also defective in the synthesis of late viral proteins (Table 6). This result is in apparent contradiction to earlier reports, which suggested that late viral protein synthesis is not dependent on viral DNA synthesis (18, 24). However, recent results of studies have shown that inhibitors of FV3 DNA synthesis such as phosphonoacetate (4, 11), cytosine arabinoside (12), and hydroxyurea (12) also depress late protein synthesis. To reconcile these contrasting results, we suggest that, as with T4 (14), qualitatively and quantitatively full expression of FV3 late proteins requires ongoing DNA replication. This requirement for ongoing DNA synthesis may partially reflect the need for amplification of the number of templates, because in the absence of DNA synthesis, an increase in the multiplicity of infection permits the synthesis of some (but not all) late proteins (24).

Recombination and genetic organization. By a series of two-factor crosses, we constructed a genetic map of FV3 that spans 77 recombinant units. Like similar maps estab-

lished for vaccinia (9, 13) and herpesvirus (2, 39), the accurate positioning of loci was impeded by the large variation in recombination frequencies obtained when identical crosses were examined in different experiments. In the case of FV3, this variation may be a consequence of mechanisms of viral DNA replication and virion assembly which involve (i) concatemer formation, (ii) multiple rounds of recombination among daughter molecules, and (iii) packaging circularly permuted and terminally redundant headfuls of viral DNA. Although tentative, the recombination map will serve as the basis for continued gene mapping by marker rescue.

The work reported here and elsewhere (4, 17, 24, 32) brings the number of *ts* mutants of FV3 to 28. This enlarged panel of mutants will be useful in examining unique two-stage and two-site strategy of DNA synthesis of FV3, the role of methylation in viral gene expression, the process of virion morphogenesis, and the role of the assembly site in late gene expression.

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