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Isolation and Genetic Characterization of Temperature-Sensitive Mutants of Vaccinia Virus WR

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One hundred temperature-sensitive mutants of vaccinia virus WR were isolated from virus that had been mutagenized with 5-bromodeoxyuridine or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. A rapid screening procedure based on the ability of vaccinia virus to form plaques under liquid overlay medium was used to identify potential mutants among randomly picked plaque isolates or plaques preselected for their small size after temperature shift-up. The preselection technique resulted in a sixfold increase in the number of successful mutant isolations relative to the number of plaques picked. All of the mutants had efficiencies of plating at 39.5°C relative to that at 33°C of 10^{-4} or less, and 33 of 40 produced 10% or less of the amount of virus at the nonpermissive temperature (39.5°C) relative to that at the permissive temperature (33°C). Experiments with the fluorescent DNA binding dye Hoechst 33258 demonstrated that 6 of the 100 mutants failed to form characteristic cytoplasmic DNA factories at 39.5°C. To facilitate the functional grouping of such a large number of mutants, a rapid infectious center assay was developed. Thirty of the mutants were assigned to 16 or 17 complementation-recombination groups by using this assay. Recombination experiments have allowed the construction of a genetic map representing 22 mutants in 12 of these groups.

Vaccinia virus, a member of the orthopoxvirus group, is a large, morphologically complex, DNA-containing virus that replicates in the cytoplasm of eucaryotic cells (reviewed by Moss [29, 30]). Several features of vaccinia virus make it a particularly suitable system in which to study virus replication and regulatory mechanisms. First, its cytoplasmic site of replication enables one to study viral DNA synthesis in the absence of the host replication machinery. Second, the virion contains a number of enzyme activities including a DNA-dependent RNA polymerase as well as enzymes for capping and methylating the 5′ end and polyadenylating the 3′ end of mRNA. The presence of these enzymes in the virus core and the ability to obtain them in soluble form (1, 30, 42) offer a unique opportunity to study mRNA transcription and modification in an in vitro system. Third, virion morphogenesis proceeds via a closely coordinated series of events including the apparent de novo synthesis of the virion lipoprotein envelope. Although this morphopoietic sequence has been studied extensively by electron microscopy (12, 28), little is known about the biochemical steps in virion assembly. Fourth, the virus replication cycle is characterized by both transcriptional (2, 10, 32, 34) and translational (18, 26) regulation of virus gene expression, while at the same time host DNA, RNA, and protein syntheses are inhibited.

Despite extensive biological and biochemical investigations of virus replication, very little is known about the functional organization of the vaccinia virus genome. Detailed restriction endonuclease maps of the viral DNA have been presented (25, 40, 47, 48), but at the present time only one of potentially 200 virus-coded polypeptides, the viral thymidine kinase, has been assigned to both a specific function and a physical location on the viral DNA (46). This report describes the isolation and partial characterization of 100 temperature-sensitive (*ts*) mutants of vaccinia virus WR that should prove to be useful tools not only for investigating virus replication and regulatory mechanisms but also for elucidating the functional organization of the vaccinia virus genome through genetic and physical mapping of their *ts* mutations. A similar approach is underway in other laboratories employing vaccinia virus strain WR (9), strain IHD-W (11), strain Copenhagen (14, 15), strain MM (7), and the closely related rabbitpox virus (23, 33, 39).

MATERIALS AND METHODS

Virus. Vaccinia virus WR was obtained from Bernard Moss (National Institutes of Health). The wild-

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type strain was plaque purified three times at the nonpermissive temperature (39.5°C) before mutant isolation to avoid repeated isolation of any spontaneous mutants that might be present in the stock.

Cells and cell culture. For most experiments and virus titrations, BSC-40 cells (B. Moss) were employed. These cells were derived from BSC-1 cells by Brockman and Nathans (3) after passage at 40°C. Monolayers of BSC-40 cells were propagated in Eagle minimal essential medium (MEM) supplemented with 5% fetal calf serum. HeLa S-3 cells (C. Milcarek, Columbia University) were used when suspension cultures were more convenient. These cells were grown in Joklik modified MEM containing 7% horse serum and twice the normal concentration of glutamine. The serum concentration was lowered to 5% before infection.

Plaque assay. The plaque assay was performed essentially as described by Salzman et al. (37). Briefly, confluent monolayers of BSC-40 cells in 60-mm tissue culture dishes (Nunc) were infected with 0.5 ml of the desired virus dilution in Dulbecco phosphate-buffered saline (PBS) without Ca^{2+} and containing 0.1% crystallized bovine serum albumin. Virus was adsorbed at 37°C for 3 h with gentle rocking of the plates every 30 min. The monolayers were then overlaid with 6 ml of MEM containing 1% Difco purified agar and 2.5% fetal calf serum. After 3 days of incubation at 33, 37, or 39.5°C, the monolayers were stained with 2.5 ml of overlay medium containing 0.01% neutral red; the plates were reincubated at the appropriate temperature until the next day, when plaques were counted. Titrations were occasionally performed under liquid overlay medium (MEM plus 2.5% fetal calf serum). In this case, the plates were incubated for 40 h after infection before staining with a 0.1% solution of crystal violet in 20% aqueous ethanol.

Dispersion of virus aggregates. Virus inocula and all samples to be titrated for infectious virus were sonicated four times for 15 s with an MSE sonicator immediately before use to disperse virus clumps and to release virus from infected cells. However, mutant stocks and all inocula for virus preparations that were to be passed serially were never sonicated directly to prevent possible cross-contamination of the mutants. Instead, infected cells were frozen and thawed three times to release virus, and inocula were treated with an equal volume of 0.25% trypsin in PBS for 30 min at 37°C (37).

Temperature of incubation. We selected 33°C as the permissive temperature and 39.5°C as the nonpermissive temperature; the wild-type virus forms plaques with equal efficiency at these two temperatures. However, the final yield of virus in infected HeLa or BSC-40 cells at 39.5°C is approximately 50% of that produced at 33°C. When high multiplicities of infection (over 15 PFU per cell) are used at 39.5°C, the yield is further reduced relative to that at 33°C.

Monolayer cultures were incubated in Forma water-jacketed incubators in a 5% CO_2-in-air atmosphere. Temperatures were controlled to ±0.2°C. Suspension cultures were incubated in water baths maintained to ±0.1°C by Braun Thermomix circulating thermoregulators.

Mutagenesis. Both 5-bromodeoxyuridine (BUdR) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) were used to mutagenize the wild-type virus as it was replicating. Monolayers of BSC-40 cells were infected with 5 to 10 PFU per cell. After a 1-h adsorption period, the monolayers were washed twice with PBS, overlaid with fresh medium containing the desired concentration of mutagen, and incubated at 33°C for 24 h. The cells were scraped from the dishes, centrifuged, and suspended in fresh medium. The final yields of virus were determined by using the plaque assay. Concentrations of BUdR from 0.5 to 5 μg/ml reduced the final yield of virus to 10^{-2} to 10^{-4}. Similar results for yield reduction and mutant isolation were obtained whether the experiments were performed in a darkened room or with illumination from a yellow bulb. NTG at concentrations from 3 to 10 μg/ml reduced the final yield of virus by 50 to 75%. Several independent cultures were included for each concentration of mutagen to reduce the possibility of isolating sister mutants.

Isolation of mutants. (i) Random screening. Virus that had replicated in the presence of BUdR or NTG was inoculated onto monolayers of BSC-40 cells to give approximately 10 to 20 plaques per plate. Well-separated plaques were picked with a Pasteur pipette and suspended in 2 ml of MEM plus 2% fetal calf serum. The method employed to screen plaques for their temperature sensitivity was based on that described by Sambrook et al. (39). Paired monolayers of BSC-40 cells in Costar 24-well tissue culture dishes were infected with 5 μl of each plaque suspension to be tested; the virus was added directly to the medium (1 ml) in which the monolayers were formed. One of each set of dishes was incubated at 33°C, and the other was incubated at 39.5°C. After approximately 40 h, the monolayers were stained with crystal violet, and the abilities of each isolate to form plaques at the two temperatures were compared. The temperature sensitivity of isolates that did not form plaques at 39.5°C in this rapid test was confirmed by the conventional plaque assay. Mutants passing this second test (about half of those passing the rapid test) were plaque purified two times at the permissive temperature and tested again for temperature sensitivity before preparation of a large-scale stock in monolayers of BSC-40 cells. All working stocks were prepared in suspension cultures of HeLa cells directly from the original large-scale stock, thus ensuring maintenance of the genotype of each mutant.

(ii) Preselection by temperature shift-up. Plaque assay plates were infected with mutagenized virus and incubated at the permissive temperature. After 48 h, the plates were shifted to 39.5°C for an additional 24 h before staining with overlay medium containing neutral red. Small plaques with indistinct edges compared with the large clear wild-type plaques were picked and tested for temperature sensitivity as described above for randomly picked plaques.

Test for DNA replication at 39.5°C. Monolayers of BSC-40 cells on glass cover slips in 35-mm tissue culture dishes were infected with the mutants or the wild type at a multiplicity of 5 to 10 PFU/cell. After 1 h at 33 or 39.5°C, unadsorbed virus was removed by washing with PBS, and 2 ml of fresh prewarmed medium was added; the dishes were returned to 33 or 39.5°C. At 4 or 8 h postinfection, the monolayers were washed with PBS, fixed with methanol-acetic acid (3:1, vol/vol), and stained with 0.5 μg of Hoechst 33258 (American Hoechst) per ml as described by

TABLE 1. Frequency of *ts* mutant isolation

Mutagen	Concn (μg/ml)	No. of mutants/ no. of plaques picked (%)	Mutant numbers
Randomly picked plaques			
BUdR	0.5	1/58 (1.7)	*ts*12
	1.0	12/426 (2.8)	*ts*1–4, 9, 13, 16, 17, 34–37
	2.0	10/685 (1.5)	*ts*6, 8, 10, 14, 18, 19, 40–42
	3.0	2/49 (4.1)	*ts*15, 20
	5.0	5/517 (0.97)	*ts*5, 7, 11, 31–33
NTG	3.0	2/248 (0.81)	*ts*26,30
	6.0	8/202 (4.0)	*ts*21–25, 27–29
Test of preselection technique[a]			
NTG, preselected	5.0	28/52 (54)	*ts*38, 39, 43–60, 62, 63, 93–96, 98, 99
	10	>10/66[b]	*ts*64–70, 97, 100, 101
NTG, not preselected	5.0	13/149 (8.7)	*ts*76, 80–89, 91, 92
	10	>9/118[b]	*ts*71–75, 77–79, 90

[a] Plaques were preselected on the basis of their small size after temperature shift-up.

[b] Not all potential mutants from the cultures treated with 10 μg of NTG per ml identified in the spot test were analyzed further. Therefore, only a minimum value for the frequency of *ts* mutant isolation is given.

Esteban (17). The cover slips were inverted onto microscope slides in a drop of glycerol-citric acid phosphate buffer (1:1) (pH 4.1) and examined in a fluorescence microscope.

Complementation. (i) Suspension culture. The time course of complementation was followed in suspension culture. HeLa cells at a concentration of 10^7/ml were infected with 3 PFU of each of two mutants per cell in pairwise crosses or with 6 PFU of a single mutant or the wild type. After 30 min of adsorption at 39.5°C, the cells were centrifuged, washed with prewarmed medium, suspended at 4×10^5 cells per ml, and returned to 39.5°C. Samples were taken at various times and frozen immediately until the end of the experiment, at which time they were all assayed by the plaque assay at 33°C. Complementation indexes were calculated according to the following formula: [yield

TABLE 2. Properties of *ts* mutants of vaccinia virus WR

Mutant	EOP 39.5°C/ 33°C	Leakiness[b] yield 39.5°C/ 33°C × 100 (%)	Small plaques at 39°C		DNA synthesis[e] at 39.5°C
			Size[c]	Frequency (%)[d]	
*ts*1	2.5×10^{-5}	4.6	M	20	+
*ts*2	$<6.0 \times 10^{-6}$	3.8	S	10	+
*ts*3	$<6.9 \times 10^{-6}$	4.0	S	10–30	+
*ts*4	$<8.1 \times 10^{-7}$	0.48	–		+
*ts*5	$<1.2 \times 10^{-4}$	0.91	M	10	+
*ts*6	$<2.9 \times 10^{-7}$	2.8	–		+
*ts*7	$<1.9 \times 10^{-6}$	4.3	VS	1–10	+
*ts*8	$<2.9 \times 10^{-5}$	6.1	VS	0.1	+
*ts*9	$<7.1 \times 10^{-5}$	3.9	VS	1–10	+
*ts*10	$<9.8 \times 10^{-5}$	14	S	10–30	+
*ts*11	$<1.1 \times 10^{-4}$	12	S	10–30	–
*ts*12	$<1.2 \times 10^{-4}$	9.6	–		–
*ts*13	$<1.8 \times 10^{-4}$	9.0	–		+
*ts*14	$<1.4 \times 10^{-4}$	1.6	–		+
*ts*15	$<1.5 \times 10^{-5}$	10	S	10–30	+
*ts*16	$<3.9 \times 10^{-6}$	0.92	–		+
*ts*17	$<1.0 \times 10^{-6}$	2.7	–		+
*ts*18	$<7.4 \times 10^{-7}$	0.33	–		+
*ts*19	$<6.6 \times 10^{-6}$	6.5	S	1–10	+
*ts*20	$<7.3 \times 10^{-6}$	0.6	–		+
*ts*21	1.2×10^{-5}	2.7	–		+
*ts*22	$<1.3 \times 10^{-5}$	4.9	S	1–10	+
*ts*23	$<8.1 \times 10^{-7}$	1.3	–		+
*ts*24	$<5.6 \times 10^{-6}$	1.8	S	10–20	+
*ts*25	$<4.6 \times 10^{-5}$	17	VS	25	+
*ts*26	$<2.5 \times 10^{-5}$	13	VS	25	+
*ts*27	$<5.0 \times 10^{-6}$	9.3	VS	10	+
*ts*28	$<7.6 \times 10^{-5}$	5.7	M	20	+
*ts*29	$<2.6 \times 10^{-7}$	23	–		+
*ts*30	$<5.2 \times 10^{-7}$	0.07	–		+
*ts*31	$<2.3 \times 10^{-6}$	27	S	25	+
*ts*32	2.4×10^{-5}	0.02	–		+
*ts*33	$<3.8 \times 10^{-5}$	12	S	25	+
*ts*34	$<5.0 \times 10^{-5}$	2.7	S	10	+
*ts*35	$<7.4 \times 10^{-5}$	2.5	–		–
*ts*36	$<1.0 \times 10^{-4}$	2.4	–		+
*ts*37	$<2.3 \times 10^{-5}$	8.0	VS	10	+
*ts*38	3.5×10^{-6}	ND[f]	–		+
*ts*39	$<2.8 \times 10^{-5}$	ND	VS	10	+
*ts*40	$<4.8 \times 10^{-5}$	0.36	–		+
*ts*41	8.1×10^{-6}	0.50	–		+
*ts*42	$<1.2 \times 10^{-5}$	1.7	–		+
*ts*43	$<4.0 \times 10^{-7}$	ND	–		+
*ts*44	7.0×10^{-6}	ND	–		+
*ts*45	3.8×10^{-5}	ND	–		+
*ts*46	$<5.0 \times 10^{-5}$	ND	–		+
*ts*47	$<7.7 \times 10^{-6}$	ND	–		+
*ts*48	1.0×10^{-5}	ND	–		+
*ts*49	3.3×10^{-6}	ND	–		+
*ts*50	1.8×10^{-5}	ND	–		+
*ts*51	3.2×10^{-5}	ND	–		+
*ts*52	3.2×10^{-6}	ND	–		+
*ts*53	$<2.9 \times 10^{-5}$	ND	S	10	+
*ts*54	$<2.0 \times 10^{-5}$	ND	S	10	+
*ts*55	4.2×10^{-6}	ND	S	1	+
*ts*56	$<9.1 \times 10^{-7}$	ND	–		+
*ts*57	6.0×10^{-6}	ND	–		+
*ts*58	6.1×10^{-7}	ND	–		+

TABLE 2.—*Continued*

Mutant	EOP 39.5°C/33°C	Leakiness[b] yield 39.5°C/33°C × 100 (%)	Small plaques at 39°C		DNA synthesis[e] at 39.5°C
			Size[c]	Frequency (%)[d]	
*ts*59	5.9×10^{-6}	ND	–		+
*ts*60	$<3.6 \times 10^{-5}$	ND	M	10	+
*ts*62	3.4×10^{-6}	ND	–		+
*ts*63	3.0×10^{-6}	ND	–		+
*ts*64	6.3×10^{-7}	ND	–		+
*ts*65	$<5.3 \times 10^{-7}$	ND	–		–
*ts*66	$<7.1 \times 10^{-7}$	ND	–		+
*ts*67	1.4×10^{-5}	ND	–		+
*ts*68	$<5.0 \times 10^{-6}$	ND	–		+
*ts*69	2.6×10^{-6}	ND	–		–
*ts*70	$<2.4 \times 10^{-6}$	ND	–		+
*ts*71	$<1.0 \times 10^{-6}$	ND	–		+
*ts*72	$<1.2 \times 10^{-6}$	ND	–		+
*ts*73	$<8.3 \times 10^{-6}$	ND	–		+
*ts*74	$<4.0 \times 10^{-7}$	ND	–		+
*ts*75	4.6×10^{-6}	ND	–		+
*ts*76	1.3×10^{-5}	ND	–		+
*ts*77	$<2.8 \times 10^{-7}$	ND	–		+
*ts*78	2.3×10^{-5}	ND	–		+
*ts*79	$<4.0 \times 10^{-5}$	ND	S	10	+
*ts*80	$<1.1 \times 10^{-5}$	ND	–		+
*ts*81	$<2.0 \times 10^{-5}$	ND	S	10	–
*ts*82	$<1.7 \times 10^{-6}$	ND	–		+
*ts*83	$<2.8 \times 10^{-5}$	ND	VS	1–10	+
*ts*84	$<6.2 \times 10^{-6}$	ND	–		+
*ts*85	3.5×10^{-6}	ND	–		+
*ts*86	$<5.6 \times 10^{-7}$	ND	–		+
*ts*87	$<2.8 \times 10^{-7}$	ND	–		+
*ts*88	2.1×10^{-5}	ND	–		+
*ts*89	$<4.2 \times 10^{-6}$	ND	–		+
*ts*90	9.2×10^{-6}	ND	–		+
*ts*91	$<1.7 \times 10^{-5}$	ND	S	10–30	+
*ts*92	$<1.3 \times 10^{-5}$	ND	VS	10	+
*ts*93	$<2.8 \times 10^{-7}$	ND	–		+
*ts*94	$<1.6 \times 10^{-7}$	ND	–		+
*ts*95	$<2.2 \times 10^{-5}$	ND	VS	10	+
*ts*96	$<1.6 \times 10^{-5}$	ND	S	10	+
*ts*97	$<3.2 \times 10^{-7}$	ND	–		+
*ts*98	$<6.2 \times 10^{-5}$	ND	S	1	+
*ts*99	$<2.1 \times 10^{-5}$	ND	M	10	+
*ts*100	$<3.4 \times 10^{-7}$	ND	–		+
*ts*101	6.3×10^{-7}	ND	–		+
Wild type	1.0	50			

[a] Monolayers of BSC-40 cells were infected at 33°C and the final yield of virus was assayed at 33 and 39.5°C. Only large plaques (1 to 2 mm) were counted at 39.5°C. In most cases only a maximum value is given because of the cytotoxicity of low dilutions of the mutants.

[b] Monolayers of BSC-40 cells were infected with 5 PFU per cell and incubated at 33 and 39.5°C. The final yield of virus was titrated at 33°C. The values given are the means of two to four determinations.

[c] The diameter of small plaques formed by some of the mutants at 39.5°C was measured: M, medium (0.5 to 1 mm); S, small (0.2 to 0.4 mm); VS, very small (<0.1 mm); –, no small plaques were formed. The diameter of wild-type plaques at 39.5°C is 1.5 to 2 mm.

(A + B) titrated at 33°C]/(yield A or B titrated at 33°C, whichever is higher).

(ii) Monolayer culture. Confluent monolayers of BSC-40 cells in 35-mm tissue culture dishes were infected with mutants singly or in pairs as described above. After 1 h at 39.5°C, unadsorbed virus was removed by washing two times with PBS. Fresh prewarmed medium was added, and the monolayers were incubated at 39.5°C for 24 h. The final yield of virus was assayed by the plaque assay at 33°C, and the complementation indexes were calculated as before.

Infectious center assay for functional groups. Monolayers of BSC-40 cells in 24-well dishes were infected with 5 PFU of each of two mutants per cell or with 10 PFU of a single mutant or the wild type. After 1 h of adsorption at 39.5°C, the monolayers were washed with PBS. The cells were then removed from the dish by treatment with 0.04% trypsin (GIBCO Laboratories; 1:300) in PBS containing 0.5 mM EDTA. The infected cells were diluted in MEM containing 5% fetal calf serum, and approximately 200 cells were plated onto preformed monolayers of BSC-40 cells in 60-mm dishes. After 4 h at 33 or 39.5°C to allow the cells to attach, the monolayers were overlaid with agar as described above for the plaque assay and incubated at the appropriate temperature. The ability of the doubly infected cells to form plaques at the two temperatures was compared and expressed as the efficiency of plating (EOP) at 39.5°C relative to that at 33°C (EOP 39.5°C/33°C) × 100. The EOP of wild type-infected cells at both temperatures was 75 to 100%. Cells infected with a single mutant at 39.5°C formed no plaques or the characteristic small plaques associated with some of the mutants.

Recombination. BSC-40 cells were infected with pairs of mutants essentially as described above for complementation in monolayer culture, except that 5 PFU of each mutant per cell was used in crosses and 10 PFU per cell was used for the singly infected controls. The monolayers were incubated at 33°C for 24 h, and titers of the final yield of virus were determined at 33 and 39.5°C. Recombination frequencies were calculated according to the following formula: [yield (A × B) titrated at 39.5°C × 2 × 100]/[yield (A × B) titrated at 33°C]. The level of reversion determined from singly infected controls proved to be insignificant in calculating recombination frequencies.

RESULTS

Isolation of mutants. One hundred *ts* mutants of vaccinia virus WR which replicate with reduced efficiency at 39.5°C relative to 33°C were isolated by the procedures described above. Both BUdR and NTG were effective in inducing

[d] The frequency of small plaques relative to the number of plaques formed at 33°C was estimated for each mutant.

[e] DNA synthesis was measured with the fluorescent DNA binding dye Hoechst 33258. +, Formation of cytoplasmic DNA factories at 39.5°C, similar to wild type; –, no or greatly reduced DNA factories at 39.5°C.

[f] ND, Not done.

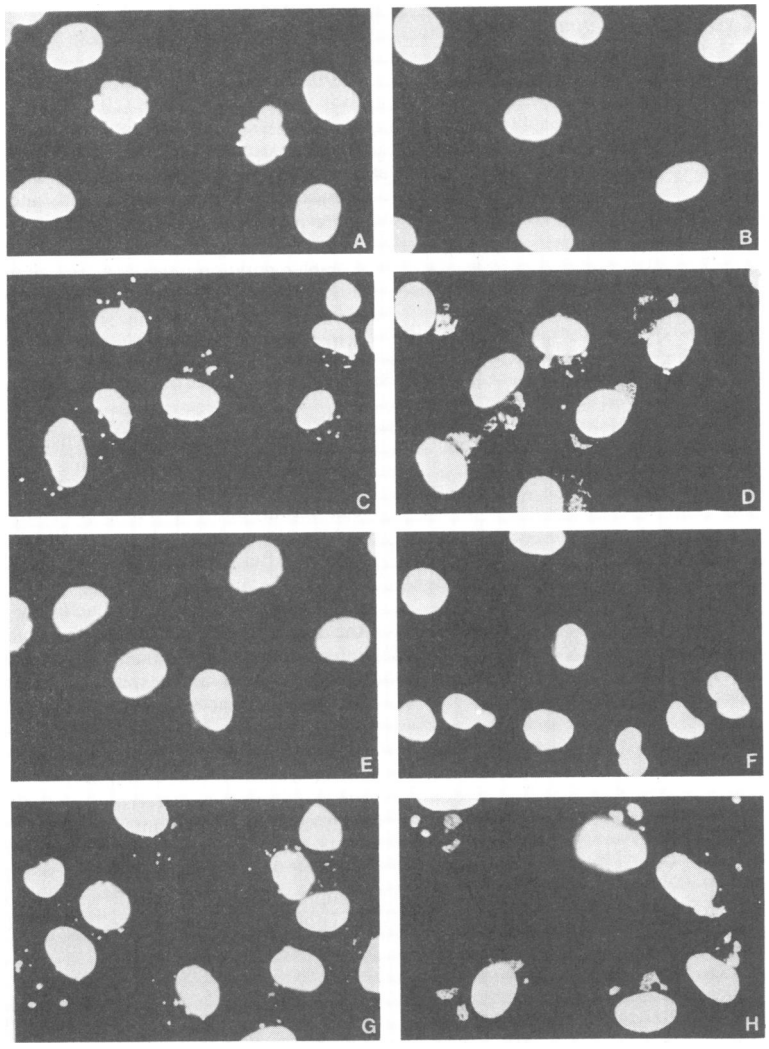

FIG. 1. Formation of viral cytoplasmic DNA factories by *ts* mutants of vaccinia virus. Monolayers of BSC-40 cells were infected with the wild type or *ts*12 and stained with the fluorescent DNA binding dye Hoechst 33258 at 4 or 8 h after infection as described in the text. (A) Mock-infected cells at 39.5°C; (B) cells infected with the wild type at 39.5°C in the presence of 5 mM hydroxyurea and stained at 8 h postinfection; (C) wild type-infected cells at 39.5°C for 4 h; (D) Wild type-infected cells at 39.5°C for 8 h; (E) *ts*12-infected cells at 39.5°C for 4 h; (F) *ts*12-infected cells at 39.5°C for 8 h; (G) *ts*12-infected cells at 33°C for 4 h; (H) *ts*12-infected cells at 33°C for 8 h.

ts mutations. The frequencies of mutant isolation for the various concentrations of mutagen employed are given in Table 1.

The first 40 mutants isolated (*ts* 1 through 37 and 40 through 42) were identified among 2,185 plaque isolates randomly picked from plaque assay plates infected with mutagenized virus. The remaining 60 mutants were isolated in a single experiment performed to test the efficacy of a modified plaque enlargement technique (preselection by temperature shift-up) in identifying *ts* plaques before picking (see above). When 5 μg of NTG per ml was used, 28 of 52 plaques preselected for their small size after temperature shift-up were *ts*, compared with 13 of 149 randomly picked plaques from cultures treated with the same concentration of mutagen (Table 1). Thus, the preselection technique resulted in a sixfold increase in the number of successful mutant isolations relative to the number of plaques picked. Approximately 1,400 plaques from the culture treated with 5 μg of NTG per ml were screened with the preselection technique so that the overall frequency of mutation to *ts* was 2%. Because of the large number of mutants obtained in this experiment, not all potential mutants from the culture treated with 10 μg of NTG per ml were studied further.

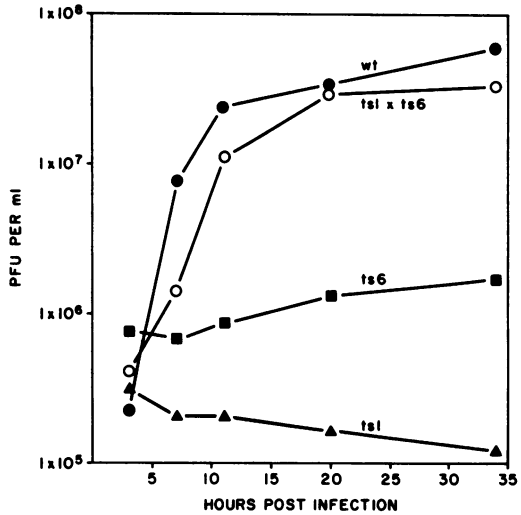

FIG. 2. Time course of complementation between *ts* mutants of vaccinia virus. Suspension cultures of HeLa cells were infected at 39.5°C with 3 PFU of each of two mutants per cell or 6 PFU of a single mutant or the wild type. Samples were taken at various times and assayed for infectious virus at 33°C by using the plaque assay. WT, Wild type.

General properties of *ts* mutants. All of the mutants had EOP 39.5°C/33°C of 10^{-4} or less (Table 2). In most cases, only the maximum values are given because of the cytotoxic effect of low dilutions of virus and the production of small plaques at 39.5°C by approximately one-third of the mutants. The size of these small plaques is characteristic of each mutant (Table 2) and ranges from minute pinpoints to about one-half the diameter of wild-type plaques. Although in most cases it was difficult to obtain an accurate count of these small plaques, an estimation of their frequency relative to the number of plaques formed at 33°C is given in Table 2. When these small plaques were picked and assayed by the plaque assay at 33°C, they were found to contain less than 1% of the amount of infectious virus found in wild type plaques; some contained no detectable virus. At the nonpermissive temperature only small plaques were again formed, indicating that the virus maintained the *ts* phenotype. A smaller proportion of the mutants isolated after preselection (9 of 38) formed small plaques than those that were isolated by random screening (26 of 62).

The leakiness of the first 40 mutants was examined in yield experiments (Table 2). Nine of the mutants produced less than 1% of the amount of virus at 39.5°C relative to that produced at 33°C, 16 produced between 1 and 5%, 8 produced between 5 and 10%, 4 produced between 10 and 15%, and 3 produced between 15 and 30%. Although most small plaque formers were leaky in yield experiments, there were some exceptions: *ts*5 and 24 formed small plaques, but were not leaky, whereas *ts*12, 13, and 29 were leaky but did not form small plaques.

Synthesis of DNA by the mutants at 39.5°C. Vaccinia virus DNA replication takes place in the cytoplasm of the infected cells in discrete areas called factories (5, 13, 20, 21). We have used a cytochemical technique employing the fluorescent DNA-binding dye Hoechst 33258 (17, 36) to visualize the cytoplasmic DNA factories in cells infected with the mutants at the nonpermissive temperature. In preliminary experiments fluorescent cytoplasmic inclusions were detected as early as 2 h after infection with the wild type at 39.5°C and 3 h after infection with the wild type at 33°C. They were completely absent in uninfected cells in which only the brightly fluorescent nucleus was visible (Fig. 1A), and their formation was completely inhibited by the inclusion of 5 mM hydroxyurea in the culture medium (17) (Fig. 1B). The appearance of these factories in wild type-infected cells at 4 and 8 h after infection at 39.5°C is shown in Fig. 1C and D. Only 6 of the 100 mutants failed to form cytoplasmic DNA factories at the nonpermissive temperature (Table 2). An example of one of these is shown in Fig. 1E and F. Fluorescent cytoplasmic inclusions were visible in less than 1% of the cells at 4 and 8 h after infection at 39.5°C, although they appeared normally at 33°C (Fig. 1G and H). It remains to be determined whether these mutants are defective in DNA synthesis per se or at some earlier step in the replication cycle such as uncoating.

Complementation. Complementation experiments were undertaken to separate the mutants into functional groups before biochemical analysis of their phenotypic defects. Because the complementation analysis of such a large number of mutants is a formidable task, standard quantitative complementation was performed with the first six mutants, and then these results were compared with those of a more rapid infectious center assay. Figure 2 shows the kinetics of virus production at 39.5°C in a suspension culture of HeLa cells mixedly infected with *ts*1 and 6 as well as in cells infected with wild-type virus or with each mutant alone. In the mixed infection, there was a significant increase in the amount of virus produced over that in the singly infected controls. The complementation indexes, based on the 30-h time points for all possible pairwise combinations of these six mutants, ranged from 0.9 to 84 (Table 3, section 1). If a complementation index of 2 or more is taken as evidence of positive complementation, two pairs of mutants failed to complement: *ts*3 and 5 (complementation index, 0.9) and *ts*3 and 6

TABLE 3. Complementation and recombination between representative *ts* mutants of vaccinia virus

Section	Cross	Complementation index[a] (%)[b]	Recombination frequency (%)	Infectious center EOP 39.5°C/33°C (%)	Complementation[c]
1	*ts*1 × *ts*2	21 (15)[b]	39	70	+
	*ts*1 × *ts*3	11	27	62	+
	*ts*1 × *ts*4	36 (22)	34	62	+
	*ts*1 × *ts*5	21	21	39	+
	*ts*1 × *ts*6	23	26	70	+
	*ts*2 × *ts*3	4.2 (19)	36	45	+
	*ts*2 × *ts*4	84 (18)	60	62	+
	*ts*2 × *ts*5	2.1 (4.4)	7.0	26	+
	*ts*2 × *ts*6	11 (30)	38	61	+
	*ts*3 × *ts*4	83 (8)	27	52	+
	*ts*3 × *ts*5	0.9 (9)	24	21	+
	*ts*3 × *ts*6	1.9 (0.15)	0.12	<0.5	−
	*ts*4 × *ts*5	10 (15)	22	38	+
	*ts*4 × *ts*6	19 (14)	18	72	+
	*ts*5 × *ts*6	3.5 (16)	21	43	+
2	*ts*1 × *ts*28	13	24	27	+
	*ts*13 × *ts*18	3.5	8.9	23	+
	*ts*8 × *ts*20	4.0	9.1	23	+
	*ts*16 × *ts*19	ND[d]	28	20	+
3[e]	*ts*8 × *ts*30	3.6	9.3	18	+
	*ts*16 × *ts*40	ND	12	18	+
	*ts*17 × *ts*42	6.2	5.2	16	+
	*ts*16 × *ts*42	4.0	7.3	12	+
	*ts*16 × *ts*30	ND	19	8.0	+
	*ts*8 × *ts*18	2.2	8.8	6.6	+
	*ts*12 × *ts*35	1.8	0.97	14	−
	*ts*11 × *ts*12	0.8	1.6	13	−
	*ts*4 × *ts*40	0.3	2.5	9.1	−
	*ts*11 × *ts*35	0.8	0.63	6.0	−
	*ts*3 × *ts*19	1.0	0.89	2.6	−
	*ts*6 × *ts*19	1.2	1.4	2.4	−
	*ts*5 × *ts*28	1.0	$<10^{-3}$	0.5	−
	*ts*7 × *ts*9	0.7	<0.02	<0.5	−
	*ts*1 × *ts*10	1.1	2.7	<0.5	−
	*ts*8 × *ts*13	2.3	1.9	5.7	?

[a] Complementation was performed in suspension cultures of the HeLa cells (section 1 in the table) or monolayers of BSC-40 cells (sections 2 and 3 in the table) as described in the text. The values given are the means of two to four experiments.

[b] The numbers in parentheses represent the percentages of wild-type recombinants in the complementation yields, determined by assaying the yield at 39.5°C and at 33°C.

[c] Based on the results of all three tests: standard complementation, recombination, and the infectious center complementation assay.

[d] ND, Not done.

[e] Additional pairs in this category, including *ts*21 × *ts*34 and all pairwise combinations of *ts*14, 18, 20, 30, and 41, had EOP 39.5°C/33°C of less than 2%, complementation indexes less than 1.2, and recombination frequencies less than 0.8%.

(complementation index, 1.9). For a third pair, *ts*2 and 5, complementation was very weak (complementation index, 2.1). If *ts*2, 3, 5, and 6 are in the same complementation group, one would not expect the following pairs of mutants to complement: *ts*2 and 3, *ts*2 and 6, and *ts*5 and 6. However, complementation was observed between these pairs (Table 3, section 1), so that these mutants could not be grouped by complementation alone. An additional problem in interpreting these results arises from the high percentage of wild-type recombinants in some of the complementation yields (Table 3, section 1). In the absence of compelling evidence, the fact that the recombination frequencies were high for all crosses involving these mutants except *ts*3 × *ts*6 (Table 3, section 1) suggested placing the first six mutants into five complementation groups with *ts*3 and 6 belonging to the same group.

TABLE 4. Complementation-recombination groups of *ts* mutants of vaccinia virus WR

Mutants in the following group:																
A	B	C	D	E	F	G	H[a]	I	J	K	L	M	N	O	P	Q
*ts*1	*ts*2	*ts*3	*ts*4	*ts*5	*ts*7	*ts*8	*ts*11	*ts*13	*ts*14	*ts*15	*ts*16	*ts*17	*ts*21	*ts*24	*ts*37	*ts*42
*ts*10		*ts*6	*ts*40	*ts*28	*ts*9		*ts*12		*ts*18				*ts*34			
		*ts*19					*ts*35		*ts*20							
									*ts*30							
									*ts*41							

[a] An additional DNA$^-$ mutant, *ts*69, failed to complement in the standard test with the three mutants in group H. However, it has not been crossed with most of the other mutants.

Functional grouping by infectious center. The rapid test for functional grouping that we selected is based on an infectious center assay (see above). Briefly, BSC-40 cells were infected with pairs of mutants and then plated onto preformed monolayers of uninfected cells at both the permissive and nonpermissive temperatures. The results are expressed as the percentage of doubly infected cells that form plaques at 39.5°C relative to 33°C (EOP 39.5°C/33°C). Cells infected with pairs of mutants that complemented well in suspension culture had EOP 39.5°C/33°C in the infectious center test between 38 and 72% (Table 3, section 1). This value was somewhat lower for *ts*2 × *ts*5 (26%) and *ts*3 × *ts*5 (21%), whereas cells mixedly infected with *ts*3 and 6 did not form any plaques at 39.5°C. Thus, the infectious center test gave the same grouping that was suggested by the recombination experiments.

To further characterize the infectious center test, the phenotype of the virus present in the plaques formed by the mixedly infected cells was determined. Well-separated plaques were picked from both the 33 and 39.5°C assay plates from representative mixed infections, and titers were determined at both temperatures. Twenty-two of 27 plaques originating from the 39.5°C assay plates were found to contain at least 50% wild-type recombinants (EOP 39.5°C/33°C, >50%), whereas only 5 of 34 plaques isolated from the 33°C plates contained more than 50% wild-type recombinants. The infectious center assay is thus measuring complementation or recombination or both. Because mutants in the same complementation group are expected to recombine with low frequency, whereas those in different groups recombine with higher frequency, the infectious center test should still be valid for assigning mutants to functional groups. Situations in which this assumption might not be valid are discussed below.

We have now performed all 435 possible crosses between 30 of the first 40 mutants and have tentatively identified 16 or 17 complementation-recombination groups (Table 4). Since there was no distinct boundary between positive and negative reactions, all pairs that had EOP 39.5°C/33°C of less than 30% were analyzed by standard complementation or recombination or both. All 31 pairs that had EOP 39.5°C/33°C between 20 and 30% were confirmed to be in different groups (e.g., Table 3, sections 1 and 2). Twenty-eight pairs had EOP 39.5°C/33°C of less than 20% (Table 3, section 3). Of these, 21 pairs failed to complement in the standard test and recombined with low frequency. Six of the remaining seven pairs were tentatively assigned to different groups on the basis of their ability to complement or to recombine with frequencies greater than 5% or both. In these six pairs, one or both of the mutants consistently gave low EOP 39.5°C/33°C with other mutants and were shown to inhibit the formation of plaques by the wild type in mixed infections in an infectious center assay at 39.5°C (data not shown). The two mutants in the remaining pair, *ts*8 and 13, could not be assigned to one or two groups by any of the three tests employed.

Recombination. One of our goals is to construct a linkage map of the *ts* loci defined by our mutants by using the technique of genetic recombination (see above). In control experiments, we considered the possibility that variation in the particle/infectious unit ratio of the mutants might influence recombination frequencies if the genomes of the noninfectious particles are capable of participating in genetic interactions. Recombination frequencies were unchanged when the multiplicity of infection of the two parental viruses was varied from 2.5 to 20 PFU per cell; with a multiplicity of infection of 1, the recombination frequencies were somewhat lower as predicted by a decrease in the number of mixedly infected cells. Similarly, changing the ratio of the two mutants from 1:1 to 3:1 or 1:3 did not alter recombination frequencies significantly.

To demonstrate that true recombination was occuring in our system and that the plaques appearing on the 39.5°C plaque assay plates were not the result of complementation or leakage, five putative wild-type recombinants were picked from a number of crosses and assayed at 33°C and 39.5°C; all exhibited EOP 39.5°C/33°C between 0.80 and 1.1, which is the normal range for wild-type plaques. Since cells coinfected with two mutants in the infectious center test

TABLE 5. Reproducibility of recombination frequencies

Cross	Recombination frequencies (%)			
	Expt 1	Expt 2	Expt 3	Mean
ts2 × *ts3*	32	34	36	34
ts2 × *ts5*	8.6	8.3	7	8.0
ts2 × *ts6*	35, 40	28	38	35
ts3 × *ts5*	17	28	24	23
ts3 × *ts6*	0.10	0.12	0.12	0.11
ts5 × *ts6*	31	ND[a]	21	26

[a] ND, Not done.

were shown to occasionally produce plaques at the nonpermissive temperature that upon replaquing had EOP 39.5°C/33°C within this range, two types of artificial mixing experiments were performed to provide additional evidence for true recombination: (i) two mutants were sonicated and diluted separately and then used to coinfect plaque assay plates at multiplicities of infection similar to those expected of the two parents in the assays of the true cross; and (ii) monolayers of cells infected singly with either of two mutants were pooled after being scraped from the dishes, frozen and thawed three times to promote aggregation, and then assayed at 39.5°C with and without sonication. "Recombination frequencies" in both types of mixtures were at least 10-fold lower than in the true cross, and putative wild-type recombinants picked from the 39.5°C assay plates of the artificial mixtures had EOP 39.5°C/33°C of between 0.22 and 0.72.

Construction of a map. We have now performed most of the crosses involving 30 mutants that have been assigned to complementation-recombination groups. Although recombination frequencies varied from experiment to experiment (Table 5), reliable distances were obtained by averaging the results of several experiments. Twenty-two mutants in 12 complementation groups could be arranged in a linear order (Fig. 3). The total length of the map, obtained by summing the distances between adjacent markers, is 81.1 recombination units. Initially, several independent linkage groups such as the one shown in Table 6 were identified, and the composite map was constructed when these linkage groups were found to be linked to one another. It should be pointed out, however, that relying on recombination frequencies of greater than 20% between adjacent markers can lead to erroneous groupings; the longest distance between adjacent markers in the map in Fig. 3 is 13 recombination units. Although there are a number of anomalous results that cannot be explained at the present time, the following general observations can be made from the data presented in Fig. 3 and Tables 3, 5, and 6: (i) recombination in poxviruses is a very efficient process; (ii) mutant pairs that failed to complement in the standard test recombined with low frequency; (iii) mutations representing the same complementation-recombination group map equidistantly from more distant markers; and (iv) over short distances recombination frequencies appear to be linear and additive, whereas over longer distances the sum of the distances between adjacent markers is greater than the measured distance between the two extreme markers. The basic sequence of the map has recently been confirmed by marker rescue experiments (Ensinger and Rovinsky, manuscript in preparation).

DISCUSSION

This report describes the isolation and partial characterization of 100 *ts* mutants of vaccinia virus WR. It is possible, considering the large number of mutants in our collection and the frequencies with which mutants were isolated, that one or more mutants may contain multiple *ts* lesions. The complementation and recombination behavior of 30 mutants suggests that each contains only a single *ts* mutation. Although in a number of instances more than one mutant was isolated from a single stock of mutagenized virus, the ability of these potential siblings to recombine demonstrated that they arose after independent mutagenic events.

The first 40 mutants were identified by screening randomly picked plaque isolates for their temperature sensitivity. In later experiments, a modification of the plaque enlargement technique in which plaques were preselected for their small size after temperature shift-up greatly facilitated mutant isolation. This preselection technique had the added advantage that fewer of the mutants isolated after preselection formed small plaques at the nonpermissive temperature. However, the plaque enlargement technique may yield a more limited population of mutants if (i) late viral proteins or defective virions cause cell killing at the periphery of the plaque, causing it to enlarge; (ii) surrounding cells become infected through direct cell-to-cell spread of subviral particles; or (iii) leaky mutants cause sufficient plaque enlargement to be missed. Although this last point could be considered an advantage, we have chosen not to completely eliminate leaky mutants from our collection; it may not be possible to isolate tight mutants in some viral functions if (i) a cellular function is partially able to substitute for the *ts* viral function, or (ii) the nature of the viral function itself precludes the isolation of tight mutants. The plaque enlargement technique was used in the

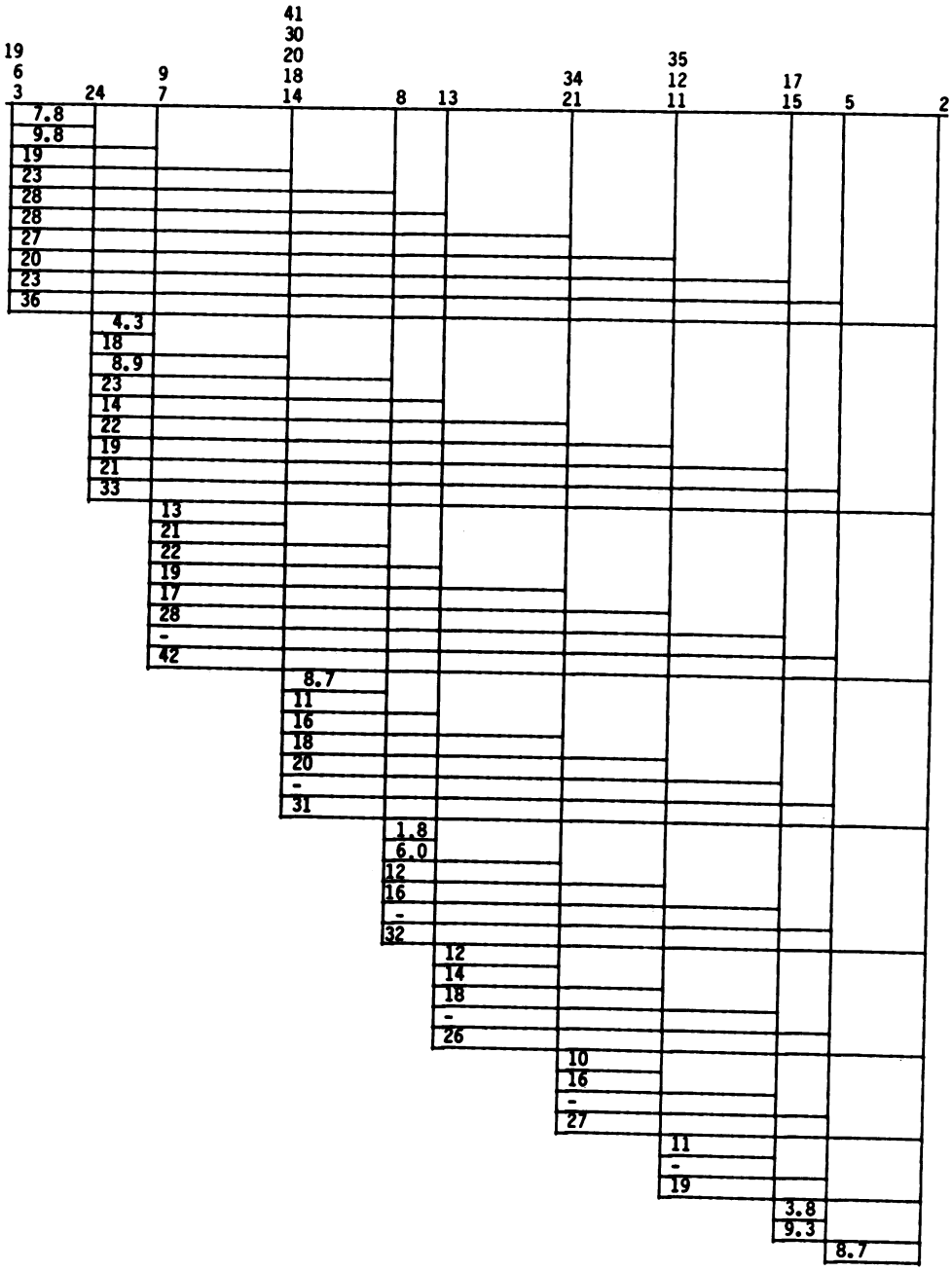

FIG. 3. Recombination map of *ts* mutants of vaccinia virus. Recombination was performed in monolayers of BSC-40 cells at 33°C as described in the text. When there was more than one marker at a locus, at least two were used in crosses with other mutants; the distances given are the mean recombination frequencies for all crosses that have been performed between markers at any two loci. *ts*15 and *ts*17 are in separate complementation-recombination groups, but their placement with respect to the mutations on either side has not been determined unambiguously. In all other cases, mutants listed above one another are in the same group.

isolation of *ts* mutants of vaccinia virus by Condit and Motyczka (9) and Chernos et al. (7).

Only six of the mutants isolated had a DNA-negative (DNA^-) phenotype as defined by their inability to form cytoplasmic DNA factories at the nonpermissive temperature (Fig. 1, Table 2). Four of these were shown to be in the same complementation group (Table 4). A similar pro-

TABLE 6. Recombination frequencies for all pairs of mutants in groups B, H, K, M, and N[a]

Group	Mutant	Recombination frequency[a] in the following group:							
		B	K	M	H			N	
		*ts*2	*ts*15	*ts*17	*ts*11	*ts*12	*ts*35	*ts*21	*ts*34
B	*ts*2								
K	*ts*15	9.3							
M	*ts*17	9.4	3.9						
H	*ts*11	19	ND[b]	11					
	*ts*12	20	12	12	1.6				
	*ts*35	19	11	11	0.63	0.97			
N	*ts*21	30	14	19	ND	8.9	11		
	*ts*34	24	9.3	20	ND	9.2	12	0.24	

[a] Values given are the means of two or more determinations.
[b] ND, Not done.

portion of DNA$^-$ mutants has been found in other collections of *ts* mutants of poxviruses: 6 of approximately 100 *ts* mutants of vaccinia virus isolated by Dales and his co-workers (11, 27), 3 of 49 mutants isolated by Chernos et al. (7), 4 of 26 mutants of Condit and Motyczka (9), and 2 of 18 mutants of rabbitpox virus isolated by Sambrook et al. (39) and reclassified as DNA$^-$ by Lake and Cooper (23). Chernos et al. (7) have described a DNA$^\pm$ phenotype (19 mutants) in which the incorporation of [^{3}H]thymidine at the nonpermissive temperature was reduced by more than 30% relative to the wild type, but the significance of this partial reduction remains uncertain. As discussed by McFadden and Dales (27), it is surprising that so few of the mutants that have been isolated have a DNA$^-$ phenotype. Although one might predict that mutants with *ts* virion-associated enzyme activities, particularly those involved in the transcription and modification of mRNA, would be DNA$^-$ mutants, it is possible that *ts* enzymes are stabilized at the nonpermissive temperature by virtue of their association with other proteins or DNA within the virus core; the temperature sensitivity of this type of mutant would not be manifest until late times when newly synthesized enzyme is to be incorporated into maturing virions. Since mutants with *ts* virion-associated enzyme activities often have virions that are more sensitive to heating in vitro than the wild type (43, 45), an alternative approach to identify enzyme mutants is to screen for increased virion thermolability relative to the wild type.

Thirty of the mutants in this report have been assigned to 16 or 17 complementation-recombination groups based on the combined results of an infectious center test, standard complementation, and recombination. In a number of crosses with our mutants standard complementation was inefficient, yielding complementation indexes between 2 and 10 for pairs of mutants that recombine with high frequency and thus are probably in different cistrons. In most of these poorly complementing pairs, one or both of the mutants consistently complemented less efficiently with other mutants, and the low complementation indexes are probably the result of (i) leakiness of one of the mutants or (ii) partial dominance of one of the *ts* gene products. These latter mutants typically inhibited the replication of the wild-type virus in mixed infections at the nonpermissive temperature (unpublished results) and may be similar to certain mutants of adenovirus (6) and herpes simplex virus (19).

We have used an infectious center assay to preliminarily identify pairs of mutants that may be in the same complementation group. Similar infectious center assays have been used previously to group *ts* mutants of herpesviruses (4, 44), simian virus 40 (22), reovirus (41), and vesicular stomatitis virus (35). Although the infectious center assay is more time consuming than the spot test developed by Condit and Motyczka (9), it can be used with mutants that are not suitable for the spot test because they form small plaques or cause extensive cell killing at the nonpermissive temperature. Both tests suffer from the disadvantage that since the final result is determined after multiple rounds of replication, they cannot discriminate between complementation and recombination. Indeed, the finding that most plaques formed by doubly infected cells contain at least 50% wild-type virus suggests that recombination does play a major role in the infectious center test. Recombination was also a factor in the spot complementation tests described by Chu and Schaffer (8) for herpes simplex virus and by Edgar et al. (16) for bacteriophage T4. Since mutants defective in the same gene are expected to recombine with low frequency, whereas mutants defective in different genes generally recombine with higher frequency, the infectious center test should still be valid for assigning mutants to functional groups. There are, however, several circumstances that could lead to erroneous results: (i) low recombination frequencies between muta-

tions in proximal regions of different genes could lead to a false negative result; (ii) mutants defective in distal regions of the same gene could recombine with sufficient frequency to give a low but positive result; and (iii) DNA$^-$ mutants in different complementation groups might recombine poorly at the nonpermissive temperature to give a false-negative result. All of these exceptions involve low or negative results. Therefore, we have used the infectious center assay as a screen to identify pairs of mutants that may be in the same cistron (those with an EOP 39.5°C/33°C of <20%) and then analyzed these crosses by standard complementation and recombination.

The recombination experiments presented in this report demonstrate that recombination can be an effective tool for mapping the *ts* loci defined by these mutants. Partial recombination maps of *ts* mutations of vaccinia virus have also been presented by Chernos et al. (7), Lake et al. (24), and Drillien et al. (Abstracts of the Fifth International Congress of Virology, Strasbourg, France, 2 through 7 August 1981, p. 336). The recent adaptation of marker rescue techniques to poxviruses (31, 38, 46) (R. Condit and Motyczka, A., Abstracts of the Fifth International Congress of Virology, Strasbourg, France, 2 through 7 August 1981, p. 338) has allowed us to confirm the general order of the genetic map and to align it with respect to restriction endonuclease maps of the virion DNA (Ensinger and Rovinsky, manuscript in preparation). We are currently investigating the biochemical defects of these mutants so that the genetic and physical maps can be given functional significance.

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