Marker Rescue of Temperature-Sensitive Mutations of Vaccinia Virus WR: Correlation of Genetic and Physical Maps

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The physical map locations of 62 temperature-sensitive mutations of vaccinia virus WR have been determined by marker rescue experiments, using cloned *Hind*III fragments of wild-type DNA. Since vaccinia virus DNA is not infectious, marker rescue was performed by infecting monolayers of cells at the nonpermissive temperature with a low multiplicity of the mutant to be rescued and transfecting with calcium phosphate-precipitated recombinant DNA. Wild-type recombinants were measured by using either a direct plaque assay technique or a two-step procedure in which the final yield of virus from the transfected cells was assayed at the permissive and nonpermissive temperatures. Mutants that had been previously assigned to the same complementation-recombination group were rescued by the same *Hind*III fragment, with the exception of three mutants in one group that were rescued by either one of two adjacent fragments. A comparison between the genetic linkage map of the temperature-sensitive mutations in 30 mutants with their physical locations demonstrated that not only was the order of the genetic map correct but also recombination frequencies generally reflected actual physical distances.

Poxviruses are large DNA-containing viruses whose genomes encode sufficient information for 100 to 200 proteins. In an effort to elucidate the functional organization of the poxvirus genome, several laboratories have undertaken a genetic analysis of poxvirus replication, using temperature-sensitive (*ts*) mutants of vaccinia virus (6, 8, 12, 14-16) and the closely related rabbitpox virus (18, 22, 24). Central to the understanding of the organization of the poxvirus genome is the establishment of genetic and physical maps of the *ts* loci represented by these mutants.

Extensive genetic linkage maps have been reported for two of the mutant collections: Drilien et al. (14) have obtained a linkage map of the mutations representing 14 complementation groups that spanned 66 recombination units, whereas we have obtained a map of 22 mutations in 11 groups that spanned 81 recombination units (16). In addition, Chernos et al. (6) have mapped seven mutations in a linear array, and Lake et al. (19) have established genetic linkage among five mutations that result in similar defects in a late stage of virion morphogenesis. However, the above workers have noted all or some of the following difficulties in using genetic recombination to map *ts* mutations of poxviruses: (i) recombination frequencies can vary considerably from experiment to experiment for a given pair of markers; (ii) some markers appear to be

unlinked to the genetic map, presumably because they are located in regions of the genome for which few markers are available; (iii) often one cannot order closely linked markers with respect to the loci on either side; and (iv) although over short distances recombination frequencies appear to be linearly additive, over longer distances the measured distance between two markers is less than the sum of the distances between adjacent markers, as would be expected from the occurrence of double crossovers. In addition, although genetic recombination can be used to determine the linear order of a series of mutations and the relative distances between them, it cannot be used to deduce physical distances between markers or to determine whether the available mutants represent all portions of the genome, particularly the ends.

The recent development of marker rescue techniques for physically mapping vaccinia virus *ts* mutations (23; R. Condit and A. Motyczka, Abstr. Fifth Int. Cong. Virol., Strausbourg, France, 1981, p. 338), the vaccinia virus thymidine kinase gene (25), and strain-specific DNA sequences (21) provides an alternative approach for mapping *ts* mutations. This report describes the marker rescue of 62 *ts* mutations in our collection, using cloned *Hind*III fragments of wild-type DNA (2). In addition, the availability of the previously determined genetic map of some of these mutations permits for the first

FIG. 1. *Hind*III and *Bgl*I restriction site maps and genetic linkage map of vaccinia virus WR. The *Hind*III map was reported by Mackett and Archard (20) and Belle Isle et al. (2); the *Bgl*I map was taken from the data of DeFilippes (13). The genetic map has been reported previously (16). Mutant numbers listed above one another are in the same complementation-recombination group with the exception of *ts*15 and *ts*17, which are in different groups. Recent data have placed *ts*8 and *ts*13 in the same group.

time a comparison of the genetic and physical maps of vaccinia virus.

MATERIALS AND METHODS

Cells. BSC-40 cells (4) were propagated in Eagle minimal essential medium containing 5% fetal calf serum as described previously (16). Confluent monolayers (1×10^6 to 1.5×10^6 cells) in 35-mm tissue culture dishes (Nunc) were used for marker rescue.

Virus. The isolation of *ts* mutants of vaccinia virus WR, the preparation of virus stocks, and virus titration by plaque assay have been described in detail (16). The permissive temperature for these mutants is 33°C and the nonpermissive temperature is 39.5°C.

Recombinant DNA. Vaccinia virus *Hind*III fragments D, E, F, G, H, I, J, K, L, M, N, and O cloned in pBR322 (2) were obtained from Bernard Moss, National Institutes of Health, Bethesda, Md. Recombinant plasmid DNA was routinely prepared from amplified cultures (2 liters) of plasmid-containing *Escherichia coli* HB101, following a large-scale version of the procedure of Birnboim and Doly (3). The plasmid was further purified by CsCl-ethidium bromide density gradient centrifugation. After the ethidium bromide was removed by extraction with CsCl-saturated isopropanol, the DNA was dialyzed against 10 mM Tris-hydrochloride (pH 7.8)–10 mM NaCl–1 mM EDTA and concentrated by ethanol precipitation. The *Hind*III-A fragment cloned in cosmid pJB8 was also obtained from B. Moss (E. Jones and B. Moss, unpublished data). Cosmid DNA was prepared from unamplified cultures essentially as described for plasmid DNA. The identity of each plasmid or cosmid

preparation was confirmed by *Hind*III digestion and gel electrophoresis through 0.6% agarose, using purified vaccinia virus DNA cleaved with *Hind*III as marker. The *Hind*III restriction site map of vaccinia virus WR is given in Fig. 1 for reference.

***Hind*III digestion of DNA.** Restriction endonuclease *Hind*III (New England Biolabs) digestion of plasmid or viral DNA was carried out at 37°C for 2 h, using the buffer conditions recommended by the supplier.

Preparation of DNA precipitates. For preparation of DNA precipitates, the procedure of Graham and van der Eb (17) was used. *Hind*III-cleaved recombinant DNA was diluted in HEPES-buffered saline (in grams per liter: NaCl, 8.0; KCl, 0.37; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.125; dextrose, 1.0; hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, 5.0; pH 7.05) to a final concentration of 2 to 6 µg/ml. Carrier salmon sperm DNA was added to give a total DNA concentration of 24 µg/ml, and the DNA was precipitated by the addition of CaCl_2 to 125 mM. The precipitates were allowed to stand at room temperature for 25 to 30 min before they were added to the cells.

Marker rescue. (i) Direct plaque assay. Monolayers of BSC-40 cells were infected with 0.01 to 0.02 PFU of the mutant to be tested per cell. After 1-h adsorption period at 39.5°C, the cells were washed with phosphate-buffered saline, overlaid with 2 ml of prewarmed minimal essential medium plus 2% fetal calf serum, and returned to 39.5°C. Two hours after infection the DNA precipitate (0.167 ml) was added to the monolayers by pipetting it directly into the medium already present on the cells. After 3 h at 39.5°C, the monolayers were washed with phosphate-buffered saline, overlaid with 2 ml of prewarmed minimal essential medi-

um plus 5% fetal calf serum, and incubated at 39.5°C for 64 to 68 h. The cells were then stained with crystal violet to visualize plaques as described previously (16). Once a mutation had been assigned to a particular *HindIII* fragment in initial screening experiments, its location was confirmed in experiments in which two different concentrations of the specific fragment were used.

(ii) **Yield experiment.** Some mutations were mapped by following a two-step procedure based on that developed by Weir et al. (25) to map the vaccinia virus thymidine kinase gene. Monolayers of BSC-40 cells were infected with *ts* mutants and exposed to DNA as described above except that all incubations were performed at 33°C. At 6 h after infection, the DNA precipitates were removed by washing with phosphate-buffered saline, fresh medium was added, and the monolayers were shifted to 39.5°C for 48 h. The cells were harvested by scraping, and the final yield of virus was assayed at 33 and 39.5°C by using the plaque assay.

Complementation. Standard complementation was performed in doubly infected monolayers at 39.5°C as described previously (16). Complementation indices were calculated according to the following formula: [yield (A + B) titrated at 33°C]/[yield (A) + yield (B) titrated at 33°C]. A complementation index of 2 or more was taken as evidence for positive complementation. This formula differs from that used previously in that the sum of the yields from the two singly infected controls was used in the denominator instead of the yield from the higher of the two single infections. In the case of only one mutant pair, *ts8* and *ts13*, did the use of the more stringent revised formula change the outcome of the complementation test. We now feel that these two mutants belong in the same functional group, thus making the total number of groups 16. Because the functional grouping presented previously (16) was based in part upon an infectious-center test that does not differentiate between complementation and recombination, we have used the term complementation-recombination group. This terminology will be maintained in this report.

Recombination. Recombination experiments that use two-factor crosses and the calculation of recombination frequencies have been described before (16). The previously determined recombination map is shown in Fig. 1.

RESULTS

Marker rescue technique. Preliminary experiments with *ts4* indicated that it could be rescued by the recombinant plasmid containing vaccinia virus *HindIII* fragment I. Therefore, various parameters of the marker rescue technique were investigated with *ts4*. Although in the case of *ts4* the use of uncleaved plasmid DNA resulted in a greater number of plaques per plate compared with the use of *HindIII*-cleaved DNA, this was not often the case with other mutants. In addition, the plaques that resulted when cleaved DNA was used were always larger and easier to quantitate. Therefore, we used *HindIII*-cleaved DNA in rescue experiments. The effect of vary-

TABLE 1. Effect of plasmid DNA concentration on marker rescue of *ts4*^a

μg of plasmid DNA per plate	Avg no. of plaques per plate
0.....	0
0.017.....	1
0.034.....	7
0.083.....	13
0.17.....	40
0.34.....	103
0.67.....	208
1.0.....	>300
2.0.....	>500

^a Cells were infected with *ts4* and transfected with recombinant plasmid containing the vaccinia virus *HindIII*-I fragment as described in the text. The values given are the average of two plates.

ing the concentration of recombinant DNA on the efficiency of rescue is shown in Table 1. Rescue was demonstrated with as little as 0.034 μg of plasmid containing *HindIII*-I per plate and increased linearly up to 2 μg per plate (the highest concentration used). The efficiency of rescue with a given concentration of a specific fragment varied considerably from mutant to mutant. Therefore, we routinely used 0.34 to 1 μg of recombinant DNA per plate. The number of plaques per plate increased linearly with increasing multiplicities of infection (data not shown). A multiplicity of 0.01 to 0.02 PFU per cell allowed efficient rescue while minimizing the cytotoxic effect of the inoculum on the cell monolayers.

To demonstrate that the plaques appearing on the transfected plates contained wild-type virus, marker rescue was performed under an agar overlay (16), and 16 well-isolated plaques were picked and assayed by the plaque assay at 33 and 39.5°C. Eight of these plaques had relative efficiencies of plaquing at 39.5/33°C within the range of wild-type plaques (0.8 to 1.1), whereas the remaining plaques presumably contained variable amounts of mutant virus (Table 2).

Some of the mutants in our collection were unsuitable for marker rescue by the direct plaque assay technique because they either formed small plaques at the nonpermissive temperature or exhibited a high degree of cytotoxicity at the multiplicities required for efficient rescue. Therefore, the *ts* lesions of these mutants were mapped in yield experiments in which the final yield of virus from the transfected cells was titrated at the permissive and nonpermissive temperatures (see Materials and Methods). An input multiplicity of 0.01 PFU per cell was again chosen so that the proportion of wild-type recombinants formed in the initially infected cells would be amplified during subsequent rounds of

TABLE 2. Progeny tests on rescued virus^a

Plaque no.	10 ⁴ PFU/ml, titrated at:		
	39.5°C	33°C	39.5/33°C
1	2.2	2.4	0.92
2	1.2	1.6	0.75
3	4.3	4.7	0.91
4	0.25	1.0	0.25
5	4.2	6.1	0.69
6	4.5	5.7	0.79
7	3.1	4.4	0.70
8	1.4	1.7	0.82
9	5.1	4.9	1.0
10	7.1	7.3	0.97
11	2.0	2.3	0.87
12	4.1	3.9	1.1
13	2.1	4.9	0.43
14	2.2	4.6	0.48
15	1.9	2.6	0.73
16	3.4	4.1	0.83

^a Cells were infected with *ts4* and transfected with cloned *HindIII* fragment I, using an agar overlay medium as described in the text. After 4 days at 39.5°C, the cells were stained with neutral red. Sixteen well-isolated plaques were picked, suspended in 2 ml of medium containing 2% fetal calf serum, and titrated by the plaque assay.

replication. Representative results are presented in Table 3. Although the backgrounds due to leakiness or reversion or both varied considerably, the relative efficiencies of plating at 39.5/33°C obtained when a specific *HindIII* fragment was used ranged from 90-fold (*ts13*) to 1,100-fold (*ts24*) greater than when either salmon sperm DNA or a nonspecific fragment was used. In the case of *ts8*, 52% of the final yield of virus consisted of presumptive wild-type recombinants.

Marker rescue of *ts* mutations of vaccinia virus. The physical locations of 62 *ts* mutations in our collection have been determined by marker rescue experiments, using cloned *HindIII* fragments of wild-type DNA. Representative results of rescue experiments, using the yield technique, are shown in Table 3, whereas results from the direct plaque assay technique are shown in Table 4. Preliminary experiments with six mutants demonstrated that four of these (*ts4*, -40, -6, and -14) could be rescued by *BglI* fragment B that had been isolated after agarose gel electrophoresis of *BglI*-cleaved wild-type DNA (unpublished data). Therefore, only the six *HindIII* fragments (F, E, O, I, G, and L) that overlap the *BglI*-B fragment (13) (Fig. 1) were used. *ts4* and *ts40*, previously assigned to the same complementation-recombination group (16), were rescued by cloned *HindIII*-I; *ts6* was rescued by *HindIII*-F and *ts14* was rescued by *HindIII*-G (Table 4). *ts17* and *ts69*, both of which had been rescued previously by the isolated

BglI-A fragment, were rescued by *HindIII*-D (Table 4). We then determined the physical locations of mutations that mapped near these six mutations on the genetic map (Fig. 1). Thus, *ts3*, a mutant in the same complementation-recombination group as *ts6*, also was rescued by *HindIII*-F (Table 3). *ts9* (Table 4) and *ts24* (Table 3), both of which were closely linked to *ts3* and *ts6*, were rescued by *HindIII*-F and *HindIII*-E, respectively. Similarly, *ts21*, whose mutation mapped to the left of the *ts17* mutation on the genetic map, was rescued by *HindIII*-H (Table 4), the first fragment to the left of *HindIII*-D on the physical map. *ts8* and *ts13*, whose mutations mapped between those of *ts14* and *ts21* on the genetic map, were rescued by *HindIII*-J (Table 3). Not unexpectedly, *ts16* (Table 4) and *ts1* and *ts10* (data not shown), three mutants that appeared to be unlinked to the genetic map, were rescued by the very large *HindIII*-A fragment.

TABLE 3. Marker rescue by yield experiment of vaccinia virus *ts* mutations by cloned *HindIII* fragments of wild-type DNA

Mutant	<i>HindIII</i> fragment	PFU/ml. ^a titrated at:		
		39.5°C	33°C	39.5/33°C × 100
<i>ts24</i>	E	1.7×10^5	3.4×10^6	5.0
	F	<67	2.4×10^6	<0.0028
	I	<67	1.5×10^6	<0.0045
	K	<67	3.9×10^6	<0.0017
	O	<67	2.7×10^6	<0.0025
	SS ^b	<67	1.5×10^6	<0.0038
<i>ts3</i>	E	$<1.0 \times 10^3$	1.6×10^7	<0.0065
	F	4.0×10^5	7.0×10^6	5.7
	SS	$<1.0 \times 10^3$	8.9×10^6	<0.011
<i>ts8</i>	H	67	6.5×10^4	0.10
	J	4.5×10^5	8.7×10^5	52
	L	4.0×10^2	3.5×10^5	0.12
	SS	1.9×10^2	2.6×10^5	0.073
<i>ts13</i>	H	<7	2.7×10^3	<0.3
	J	1.9×10^4	4.1×10^4	46
	L	<7	1.4×10^3	<0.5
	SS	<7	4.9×10^3	<0.1
<i>ts81</i>	A	1.4×10^6	4.7×10^6	30
	H	$<1.0 \times 10^3$	3.3×10^6	<0.030
	I	$<1.0 \times 10^3$	1.0×10^6	<0.10
	J	$<1.0 \times 10^3$	2.6×10^6	<0.038
	K	$<1.0 \times 10^3$	4.0×10^6	<0.025
	L	$<1.0 \times 10^3$	4.8×10^6	<0.021
	O	$<1.0 \times 10^3$	4.5×10^6	<0.022
	SS	$<1.0 \times 10^3$	4.9×10^6	<0.020

^a Monolayers of BSC-40 cells were infected with *ts* mutants and transfected with cloned *HindIII* fragments of wild-type DNA as described in the text. The final yields of virus were titrated at 33 and 39.5°C by using the plaque assay.

^b SS, Salmon sperm DNA.

TABLE 4. Marker rescue by direct plaque assay of *ts* mutations of vaccinia virus by cloned *Hind*III restriction fragments^a

Mutant	No. of plaques per plate														Salmon sperm DNA
	<i>Hind</i> III fragment														
	N	M	K	F	E	O	I	G	L	J	H	D	A		
<i>ts</i> 4				0, 0	0, 0	0, 0	50, 67	0, 0	0, 0					0, 0	
<i>ts</i> 40				0, 0	0, 0	0, 0	49, 51	0, 0	0, 0					0, 0	
<i>ts</i> 6				25, 38	0, 0	0, 0	0, 0	0, 0	0, 0					0, 0	
<i>ts</i> 14				0, 0	0, 0	0, 0	0, 0	90, 84	0, 0					0, 0	
<i>ts</i> 17									0, 0	0, 0	0, 0	60, 51		0, 0	
<i>ts</i> 69									0, 0	0, 0	0, 0	91, 69		0, 0	
<i>ts</i> 9				39, 50	0, 0	0, 0	0, 0	0, 0						0, 0	
<i>ts</i> 21								2, 1	0, 1	1, 1	161, 144	0, 2		1, 2	
<i>ts</i> 16	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	71, 95	0, 0	
<i>ts</i> 38				0, 0	0, 0	0, 0	0, 0	22, 28						0, 0	
<i>ts</i> 44				0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	64, 48	0, 0	0, 0		0, 0	
<i>ts</i> 56				0, 0	0, 1	0, 0	0, 0	0, 0	76, 77	0, 0	0, 0	0, 0		0, 0	
<i>ts</i> 66	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	119, 96	0, 0	
<i>ts</i> 88				0, 0	0, 0		0, 0	0, 0		0, 0	97, 94	0, 0		0, 0	

^a Monolayers of BSC-40 cells infected with *ts* mutants at 39.5°C were transfected with cloned *Hind*III fragments of wild-type DNA or salmon sperm DNA as described in the text.

Additional marker rescue data for some of our more recent isolates are given in Tables 3 and 4; Table 5 summarizes the physical locations of the 62 mutations that have been mapped so far. It should be noted that eight additional mutants, *ts*43, -47, -50, -54, -90, -95, -97, and -100, were not rescued by any of the 13 cloned *Hind*III fragments used in this study.

Complementation. Thirty of our mutants have been previously assigned to 16 complementation-recombination groups (16). The completion

of this analysis for such a large collection of mutants by standard complementation tests would be laborious at best. Although rapid tests such as the infectious-center test previously used in this laboratory and the spot test developed by Condit and Motyczka (8) save considerable amounts of time and effort, both suffer from the disadvantage that they do not differentiate between complementation and recombination. However, knowledge of the physical locations of the *ts* markers represented by our mutants

TABLE 5. Summary of marker rescue experiments^a

<i>Hind</i> III fragment	Size (mol wt × 10 ⁻⁶)	Mutant no.	No. of mutants	No. of complementation-recombination groups
A	30	1, 10; 16, 64; 49, 66, 84; 58; 63, 67; 74; 78; 81	13	7 to 8
D	10.3	2; 11, 12, 35, 69; 15; 17; 45; 52, 59; 93; 94; 101	13	7 to 9
E	9.9	24; 37; 46; 77	4	4
F	8.9	3, 6, 19; 7, 9; 83	6	2 to 3
G	6.0	14, 18, 20, 30, 41, 57, 73, 75, 86; 38, 51	11	2
H	5.6	21, 34, 62, 87, 88; 72	6	2 ^b
I	4.3	4, 40	2	1
J	3.2	8, 13; 44, 71, 76	5	2 ^b
K	2.9		0	
L	2.6	56; 85	2	2
M	1.4		0	
N	1.0		0	
O	0.9		0	

^a Marker rescue was performed as described in Tables 3 and 4. The molecular-weight values for vaccinia virus *Hind*III fragments were taken from Belle Isle et al. (2). *Hind*III fragments B and C were not used in this study. The mutant numbers separated by commas have been assigned to the same complementation-recombination group; those separated by semicolons are in different groups. This table is a compilation of data from Tables 6, 7, and 8, reference 16, and unpublished data.

^b *ts*72 is in the same complementation-recombination group as *ts*8 and *ts*13. Therefore, although two groups have been attributed to both *Hind*III fragments H and J, the sum of the number of groups on these two fragments is three.

TABLE 6. Complementation among *ts* mutants of vaccinia virus that were rescued by *Hind*III fragment G

Mutant	Complementation index ^a					
	<i>ts</i> 14	<i>ts</i> 41	<i>ts</i> 57	<i>ts</i> 75	<i>ts</i> 86	<i>ts</i> 38 <i>ts</i> 51
<i>ts</i> 14						
<i>ts</i> 41	0.70					
<i>ts</i> 57	0.30	0.56				
<i>ts</i> 75	0.060	0.43	0.68			
<i>ts</i> 86	0.77	0.51	0.77	1.3		
<i>ts</i> 38	5.4	31	44	51	140	
<i>ts</i> 51	7.3	25	42	80	12	0.42

^a Complementation was performed in doubly infected monolayers of BSC-40 cells as described in the text.

should greatly expedite the assignment of additional mutants to functional groups: since the *Hind*III fragments used in this study are relatively large, complementation tests need only be performed between mutants that have been assigned to the same or adjacent fragments by marker rescue experiments. Although we have not completed this analysis as yet, the data for mutations that map to selected regions of the genome are presented in Tables 6, 7, and 8. The mutations in five mutants previously assigned to complementation-recombination group J (*ts*14, -18, -20, -30, and -41) all mapped to *Hind*III-G on the physical map. Standard complementation was performed for all pairwise crosses among two of these and five of the six more recent isolates that were rescued by *Hind*III-G. The results in Table 6 demonstrate that three of the new mutants (*ts*57, -75, and -86) also belong to group J, whereas *ts*38 and *ts*51 represent a new group. *ts*73, the remaining mutant that was rescued by *Hind*III-G, was shown by the spot complementation test of Condit and Motyczka (8) to be in complementation-recombination group J (M. Ensinger and R. Rosenberg, unpublished data), thus making this group with nine members the largest in our collection. When representatives of the two groups on *Hind*III-G were paired with members of the groups on the adjacent *Hind*III fragments I and L, positive complementation was obtained in all cases (Table 7). Similar experiments, some of which are shown in Table 8, demonstrated that the 13 mutations that mapped to *Hind*III fragments H, J, and L represented five complementation-recombination groups. Two members of one complementation-recombination group (*ts*8 and *ts*13) that were rescued by *Hind*III-J failed to complement with *ts*72, whose *ts* lesion has been mapped to the adjacent *Hind*III-H fragment.

A summary of the complementation-recombination groups that have been identified so far is presented in Table 5. Mutant numbers that are separated by commas are in the same group,

whereas those separated by semicolons are in different groups. Some of the group assignments are tentative. For example, the grouping of mutants with numbers greater than 43 which were rescued by fragments A and D was based on the spot complementation test only and must be confirmed by standard complementation. Furthermore, we do not know at the present time if the group represented by *ts*1 and *ts*10 is the same or different from the other groups rescued by *Hind*III-A or whether any groups extend across two of the *Hind*III restriction sites, between fragments F and E and D and A. Therefore, we can only estimate that the 62 mutations that have been mapped by marker rescue represent a minimum of 26 and a maximum of 32 complementation-recombination groups.

Recombination. We have previously determined a linkage map of the *ts* loci of 22 mutants representing 11 complementation-recombination groups. As information concerning the physical locations of additional mutations became available, we asked whether they were genetically linked to the established linkage groups that spanned the same region of the physical map. The recombination data for one region of the genome spanning *Hind*III fragments I through H are shown in Fig. 2. The mutations representing four previously unmapped complementation-recombination groups (*ts*4 and *ts*40, *ts*38 and *ts*51, *ts*56, and *ts*44) were linked to the established genetic map in the order predicted by their physical locations. As we had observed previously, although there was generally good additivity of recombination frequencies over relatively short genetic distances, over longer distances the sum of the distances between adjacent markers was often greater than the measured distance between the two extreme markers. Thus, as more *ts* loci were added to a given interval on the genetic map, the measured

TABLE 7. Complementation among *ts* mutants of vaccinia virus that were rescued by *Hind*III fragments I, G, and L

Mutant (<i>Hind</i> III-G)	Complementation index ^a		
	<i>Hind</i> III-I, <i>ts</i> 4	<i>Hind</i> III-L	
		<i>ts</i> 56	<i>ts</i> 85
<i>ts</i> 14	17	5.9	ND ^b
<i>ts</i> 41	ND	150	25
<i>ts</i> 75	76	100	35
<i>ts</i> 86	ND	91	9.9
<i>ts</i> 38	260	690	180
<i>ts</i> 51	93	90	55

^a Complementation was performed as described in the text.

^b ND, Not done.

TABLE 8. Complementation among *ts* mutants of vaccinia virus that were rescued by *Hind*III fragments L, J, and H

Mutant	Complementation index ^a									
	<i>Hind</i> III-L		<i>Hind</i> III-J			<i>Hind</i> III-H				
	<i>ts</i> 56	<i>ts</i> 85	<i>ts</i> 8	<i>ts</i> 13	<i>ts</i> 44	<i>ts</i> 21	<i>ts</i> 62	<i>ts</i> 87	<i>ts</i> 88	<i>ts</i> 72
<i>Hind</i> III-L										
<i>ts</i> 56										
<i>ts</i> 85	220									
<i>Hind</i> III-J										
<i>ts</i> 8	5.9	2.5								
<i>ts</i> 13	7.5	3.5	1.6							
<i>ts</i> 44	140	85	4.2	15						
<i>Hind</i> III-H										
<i>ts</i> 21	+ ^b	+	2.9	11	8.6					
<i>ts</i> 62	+	+	3.8	8.0	13	0.43				
<i>ts</i> 87	+	+	4.0	3.8	4.7	0.58	0.83			
<i>ts</i> 88	+	+	3.6	4.4	3.7	0.32	0.50	0.78		
<i>ts</i> 72	+	96	1.3	0.63	55	26	30	10	15	

^a Standard complementation was performed as described in the text.

^b Positive complementation was determined by using the spot test of Condit and Motyczka (8).

length of the map in that interval increased. A revised genetic map is presented in Fig. 3. Even though no new markers have been added to the ends of the map, the total length of the revised genetic map is 128 recombination units, compared with a length of 81 recombination units for the original map. The genetic map spans *Hind*III fragments F through D on the physical map, or approximately 42% of the genome.

DISCUSSION

The physical map locations of 62 *ts* mutations of vaccinia virus WR have been determined by marker rescue experiments, using cloned *Hind*III fragments of wild-type DNA. Two marker rescue techniques were used: a direct plaque assay and a two-step yield procedure. Although the direct plaque assay technique is relatively simple and can be used to map a large number of mutations in a short time, the two-step procedure can be used with mutants that are too leaky or too cytotoxic to be mapped by the direct plaque assay technique. In addition, the two-step procedure may be more sensitive because: (i) viral replication is allowed to proceed at the permissive temperature for 6 h before a shift to the nonpermissive temperature; and (ii) wild-type recombinants are enriched in the final yield of virus through multiple rounds of replication. Although the mechanism of rescue is unknown, it is clear from the progeny tests of plaques obtained after rescue of *ts*4 (Table 2) that two processes are probably occurring: (i) recombination between the wild-type fragment and mutant DNA and (ii) complementation of *ts* virus by either wild-type recombinants or direct transcription of the wild-type fragment.

nants or direct transcription of the wild-type fragment.

The use of cloned DNA fragments circumvents the difficulty of isolating large amounts of pure restriction endonuclease fragments free of contaminating sequences. Furthermore, in our initial marker rescue experiments that used isolated *Bgl*II fragments of wild-type DNA, efficient rescue was achieved only if the wild-type fragment was coprecipitated onto the infected cells with DNA isolated from the mutant being rescued (unpublished data). A similar finding was reported by Sam and Dumbell (23). With cloned DNA fragments, however, it was possible to use at least a 50-fold-higher molar concentration of each fragment relative to when isolated fragments were used. Under these conditions coprecipitation with mutant DNA was unnecessary for efficient rescue. It is possible that coprecipitation increased the chances of a recombinational event when low amounts of the wild-type fragments were used.

Eight additional mutants in our collection were not rescued by any of the 13 cloned *Hind*III fragments. Their mutations could be located either on *Hind*III fragments B and C that were not used in this study or too close to a restriction site for efficient rescue to occur. It is also possible that some of these mutants could contain more than one *ts* lesion and would be rescued only by a mixture of fragments. Indeed, several of these mutants were suspected by the spot complementation test of Condit and Motyczka (8) not to complement mutants in two or more separate complementation-recombination groups (Ensinger and Rosenberg, unpublished data).

FIG. 2. Recombination map of *ts* mutations of vaccinia virus that map to *Hind*III-I through -H. Recombination was performed in doubly infected monolayers at 33°C as described in the text. The distances given are the mean recombination frequencies for all possible pairs of mutants at any two loci. Mutants listed above one another are in the same complementation-recombination group.

In every case but one, all members of a single complementation-recombination group were rescued by the same *Hind*III fragment. The members of the remaining group, *ts*8, -13, and -72, were shown to be rescued by the adjacent *Hind*III fragments H and J. Belle Isle et al. (2) have demonstrated that a 110K polypeptide is synthesized *in vitro* by mRNA that hybridizes to both *Hind*III-H and -J. It is tempting to speculate that the *ts* loci of these mutants lie within the gene for this 110K polypeptide, but a more detailed analysis is required to demonstrate this point conclusively.

The physical mapping of *ts* mutations presented in this paper and the earlier report of the genetic linkage map of these mutations has allowed for the first time a comparison of the genetic and physical maps of vaccinia virus (Fig. 3). Within the resolution provided by the relatively large *Hind*III fragments used in this study, the following conclusions can be drawn. (i) Recombination frequencies generally reflect physical distances accurately. (ii) The order of mutations shown on the original genetic map is correct with the exception of the loci represented by *ts*24 and *ts*7, which were reversed relative to the *ts*6 mutation. However, as more crosses were performed with these mutants, the cumulative genetic data agreed with the physical map (Fig. 3). (iii) The distribution of mutations on the physical map is not random. Although in general

larger fragments contain more *ts* mutations than smaller fragments, there are notable exceptions. For example, both *Hind*III-G (11 mutations) and *Hind*III-J (5 mutations) contain a greater number of mutations than would be expected from their size, whereas *Hind*III-E, -F, and -I contain fewer mutations than expected. Nine of the 11 mutations that map to *Hind*III-G have been assigned to a single complementation-recombination group. This cistron may represent a *ts* mutational hot spot on the vaccinia virus genome.

It is possible to make an approximation of the relationship between recombination frequency and physical distance. For example, recombination experiments have been performed between mutants in four of the complementation-recombination groups that were rescued by *Hind*III-D. The recombination frequency between the two most distant markers, obtained by summing the intervals between adjacent markers, was 28%. *Hind*III-D has a molecular weight of 10.3×10^6 (15.4 kilobase pairs) so that the observed recombination frequency per kilobase pair was 1.8%. A similar calculation based on the entire length of the genetic map (128%) which spans *Hind*III-F through -D (76 kilobase pairs) resulted in a recombination frequency of 1.7% per kilobase pair. Both estimates, however, give only a minimum value, the former because it is not known how close to the ends of the D fragment the

FIG. 3. Correlation of the *Hind*III physical map and genetic linkage map of *ts* mutations of vaccinia virus. The distances between the loci shown on the genetic map represent the mean recombination frequencies for all pairwise combinations of the mutants shown at any two adjacent loci. Mutant numbers listed below one another are in the same complementation-recombination group. Some groups contain additional mutants, but they were not included in the recombination experiments. The vertical lines connect the *Hind*III fragment on the physical map with the region of the genetic map containing mutations rescued by that fragment. *ts*5 and *ts*28 have been included in the genetic map even though they could not be rescued unambiguously by *Hind*III-D.

terminal mutations are located and the latter because we have observed that, as more markers are added to the genetic map, the total length of the map increases. It should be stressed that these calculations are actually based on the frequency of recombinants rather than the frequency of recombinational events. Therefore, they do not reveal anything about molecular mechanisms of recombination in the vaccinia virus system. The number obtained is of practical value, however, in predicting either recombination frequencies or physical distances when one of these two values is known.

As demonstrated in this paper, marker rescue of additional mutations will facilitate their characterization by the standard genetic techniques of complementation and recombination. In addition, fine-structure mapping, using smaller DNA fragments or overlapping fragments, will eventually allow the assignment of specific mutations to mRNA transcripts and polypeptides through comparison of their physical locations with transcriptional and polypeptide maps that are being generated in other laboratories (1, 2, 5, 7, 9–11, 26–28).

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