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Fine Structure Marker Rescue of Temperature-Sensitive Mutations of Vaccinia Virus within a Central Conserved Region of the Genome

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Fine structure marker rescue involving the use of subfragments of vaccinia virus *Hin*dIII DNA fragments L, J, and H has been used to map the mutations in eight temperature-sensitive mutants of vaccinia virus representing four complementation groups. Comparison of their map locations with the positions of the open reading frames and RNA transcripts that have been mapped within this region has allowed the identification of one or two polypeptides as the temperature-sensitive gene product of each mutant.

Vaccinia virus is a complex DNA-containing animal virus that replicates in the cytoplasm of infected cells. The 187-kilobase-pair (kbp) genome encodes sufficient information for 100 to 200 proteins. Although a large number of temperature-sensitive (*ts*) mutants of vaccinia virus and the closely related rabbitpox virus have been isolated (2–7, 10, 14), in only a few cases have both the function and polypeptide product of specific viral genes been identified. As one approach to characterize the *ts* mutants in our collection, we have performed fine-structure marker rescue experiments to precisely map the site of their *ts* mutations. Since investigators in a number of laboratories have mapped both mRNAs and polypeptide products that are encoded within a central conserved region of the genome (1, 9, 11, 12, 16), we have concentrated our efforts on eight mutants representing four complementation groups whose *ts* lesions have been mapped previously to adjacent vaccinia virus *Hin*dIII fragments L, J, and H (8). All of the *Hin*dIII L fragment and most of *Hin*dIII-J have recently been sequenced (9, 13, 15, 16).

The subclones of *Hin*dIII fragments L, J, and H that have been used in this study are shown in Fig. 1. Marker rescue was performed as described by Ensinger and Rovinsky (8). Briefly, monolayers of BSC-40 cells that had been infected at a concentration per cell of 0.02 PFU of the mutant to be rescued were transfected with calcium phosphate-precipitated DNA and incubated at the nonpermissive temperature (39.5°C) for 48 to 72 h. The DNA precipitates contained 6 μg of recombinant DNA and 18 μg of carrier salmon sperm DNA per ml. Marker rescue was demonstrated by the appearance of plaques on the infected cell monolayers (one-step procedure) or by assaying the final yield of virus at the permissive and nonpermissive temperatures to determine the proportion of wild-type recombinants (two-step procedure).

Two mutants, *ts*56 and *ts*85, in separate complementation groups were rescued by *Hin*dIII-L (8). *ts*56 was rescued by the L3 and 8-L4 subclones (Table 1). Thus the site of its *ts* mutation lies within a 1.5-kbp fragment from the *Xba*I site 1.7 kbp from the left end of *Hin*dIII-L to the *Rsa*I site at 3.2 kbp (Fig. 1). Although specific mRNAs have not been mapped as yet within this portion of *Hin*dIII-L, comparison of these results with the open reading frames identified by Pluciennicczak et al. (13) suggests that the *ts*56 mutation lies within one of two polypeptides with maximum predicted molecular weights of 11,000 or 41,000 (11K and 41K polypeptides) (Fig. 2). The *ts*85 mutation was mapped to a 335-base-pair fragment (clone 8-L3, Fig. 1) that lies entirely within the map positions of a late mRNA encoding a 28K polypeptide (Fig. 2) (16). Both *ts*56 and *ts*85 have virions that are more thermolabile than the wild type at 54°C (M. Ensinger, unpublished results) and thus may have mutations in virion polypeptides. The 28K polypeptide has recently been shown to be a precursor of the 25K virion core polypeptide (J. Weir and B. Moss, submitted for publication).

Five mutants representing two complementation groups were rescued by *Hin*dIII-J: *ts*44, *ts*71, and *ts*76 are in one group, and *ts*8 and *ts*13 are in the second group. A third mutant in the group with *ts*8 and *ts*13 (*ts*72) was rescued by the adjacent *Hin*dIII H fragment. *ts*44, *ts*71, and *ts*76 were all rescued by the J3 and J6 subclones (Table 2). *ts*71 was always rescued with a lower efficiency than the other two mutants in this group, and the plaques that appeared at 39.5°C were smaller than the wild type. Thus this mutant probably contains a second *ts* mutation elsewhere on the genome. Neither *ts*44 nor *ts*76 were rescued by the J7 or J8 subclones nor by the entire *Hin*dIII J fragment that had been cleaved with *Bgl*II when the one-step marker rescue technique was used. *ts*44 was rescued, albeit with lower efficiency, by J7 when the more sensitive two-step procedure was used (Table 2). These results place the *ts* lesion of *ts*44 within the 700-base-pair overlap of the J6 and J7 subclones and suggest that the *ts* mutation of *ts*76 lies very close to the *Bgl*II site 2.54 kbp from the left end of *Hin*dIII-J. Two early mRNAs that code for 21K and 41K polypeptides (open reading frames 10 and 9, respectively) are transcribed from this region (Fig. 2) (1, 11). The suggestion that the *ts*76 mutation lies closer to the *Bgl*II site than does the *ts*44 mutation makes the 21K polypeptide the more likely candidate.

Marker rescue data for the three mutants in the remaining complementation group are shown in Table 3. Both *ts*8 and *ts*13 were rescued by the J3 and J4 subclones. *ts*8 was not rescued by the J8 subclone, while *ts*13 was rescued by J8 in 4 of 13 samples from five separate experiments. Efforts to increase the efficiency of rescue of *ts*13 by J8 by coprecipitating the fragment with *ts*13 DNA were unsuccessful. We believe that the site of the *ts*13 mutation may lie within the 360-base-pair overlap of the J4 and J8 subclones, while the *ts*8 mutation lies either very close to the *Bgl*II site

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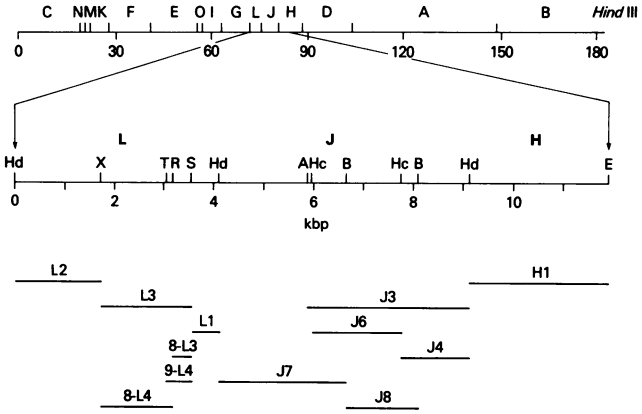

FIG. 1. Restriction endonuclease sites and recombinant DNA fragments used in marker rescue. Recombinants containing subfragments of *Hin*dIII-L, *Hin*dIII-J, and *Hin*dIII-H were constructed in plasmid or M13 (8-L3, 8-L4, and 9-L4) vectors (1, 15, 16). Restriction endonuclease sites are indicated as follows: Hd, *Hin*dIII; X, *Xba*I; T, *Taq*I; R, *Rsa*I; S, *Sal*I; A, *Ava*I; Hc, *Hin*cII; B, *Bgl*II; E, *Eco*RI. Some enzymes cleave at additional sites within this region, but only those used in the construction of recombinants are shown.

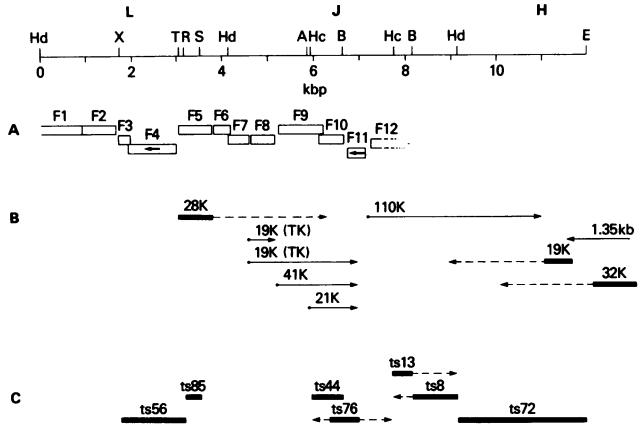

FIG. 2. Summary of open reading frames, transcripts, and physical map locations of *ts* mutations. (A) The open reading frames identified by sequence analysis of *Hin*dIII fragments L and J are redrawn from Pluciennicczak et al. (13). The direction of transcription is from left to right, with the exception of F4 and F11 which are from right to left. (B) The positions of the mRNA transcripts are taken from references 1, 9, 11, 12, and 16. The direction of transcription and the size of the polypeptide product are shown. TK denotes the mRNA for the viral coded thymidine kinase. The product of the 1.35-kilobase RNA has not been identified. Late mRNAs are diagrammed as solid lines representing the minimum size required to encode the observed polypeptide product followed by a dashed line to indicate the extent of 3′ heterogeneity (12, 16). (C) The limits of the physical locations of the *ts* mutations determined by marker rescue are shown. The mutations of *ts*8, *ts*13, and *ts*76 are probably located within the smaller solid bar but may lie within the larger region indicated by the dashed line. The abbreviations for restriction endonucleases are defined in the legend to Fig. 1.

4.0 kbp from the left end of *Hin*dIII-J or farther to the right of it. The third member of this complementation group, *ts*72, was rescued by the leftmost 2.85 kbp of *Hin*dIII-H, from the *Hin*dIII site to the first *Eco*RI site (Table 3). A single early mRNA that encodes a 110K polypeptide has been mapped to this region (Fig. 2) (1, 12). Recent evidence from two laboratories (E. Jones, C. Puckett, and B. Moss, unpublished results; R. Moyer, personal communication) suggests that one of the high-molecular-weight subunits of the virion DNA-dependent RNA polymerase is the product of this

TABLE 1. Marker rescue of *ts*56 and *ts*85 by subclones of *Hin*dIII-L[a]

Mutant	DNA	No. of plaques/plate for one-step procedure	PFU/ml titrated for two-step procedure at:		
			33°C	39.5°C	39.5/33°C
*ts*56	L	50, 78	1.2×10^7	6.1×10^6	0.51
	L1	0, 0			
	L2	0, 0			
	L3	34, 52	4.1×10^6	2.4×10^6	0.58
	8-L3	nd[b]	2.0×10^1	< 7	
	8-L4	nd	2.3×10^4	1.2×10^4	0.52
	9-L4	nd	4.7×10^1	< 7	
	SS[c]	0, 0	4.0×10^1	< 7	
*ts*85	L	108, 74	2.0×10^7	1.1×10^7	0.55
	L1	0, 0	4.3×10^2	< 7	
	L2	0, 0	1.3×10^2	< 7	
	L3	1, 2	8.9×10^4	4.4×10^4	0.49
	8-L3	nd	8.1×10^3	4.2×10^3	0.52
	8-L4	nd	6.7×10^1	< 7	
	9-L4	nd	2.1×10^4	8.5×10^3	0.40
	SS	0, 0	6.0×10^1	< 7	

[a] Marker rescue was performed as a direct plaque assay at 39.5°C (one-step procedure) or as a yield experiment in which the final yield of virus at 39.5°C was titrated at 33 and at 39.5°C (two-step procedure). The DNAs used are shown in Fig. 1. When fragments were cloned in M13 vectors, the replicative form of the phage DNA was used for marker rescue.
[b] nd, Not done.
[c] SS, Salmon sperm DNA.

TABLE 2. Marker rescue of *ts*44, *ts*71, and *ts*76 by subclones of *Hin*dIII-J

Mutant	DNA[a]	No. of plaques/plate for one-step procedure	PFU/ml titrated for two-step procedure at:		
			33°C	39.5°C	39.5/33°C
*ts*44	J	31, 40			
	J3	67, 68			
	J6	71, 72	3.6×10^5	1.6×10^5	0.44
	J7	0, 4	1.0×10^5	7.4×10^4	0.74
	J8	0, 0	2.0×10^2	< 7	
	SS[b]	0, 0	3.2×10^2	< 10	
*ts*71	J	7, 11			
	J3	11, 15			
	J6	8, 0			
	J7	0, 0			
	J8	0, 0			
	SS	0, 0			
*ts*76	J	54, 69			
	J3	58, 67			
	J6	38, 33	2.4×10^5	1.2×10^5	0.50
	J7	0, 0	7	< 7	
	J8	0, 0	30	< 10	
	SS	0, 0	< 10	< 10	

[a] The DNAs used are shown in Fig. 1.
[b] SS, Salmon sperm DNA.

TABLE 3. Marker rescue of *ts*8, *ts*13, and *ts*72[a]

Mutant	DNA	PFU/ml titrated at:		Yield at 39.5°C/ yield at 33°C
		33°C	39.5°C	
*ts*8	J	2.7×10^6	1.2×10^6	0.44
	J3	1.1×10^6	5.2×10^5	0.47
	J4	8.6×10^4	2.6×10^4	0.30
	J8	9.6×10^4	7.5×10^2	7.8×10^{-3}
	SS	8.9×10^4	< 7	
*ts*13	J	3.7×10^5	1.6×10^5	0.43
	J3	3.7×10^5	1.6×10^5	0.43
	SS	2.5×10^5	< 10	
	J4	1.1×10^5	3.5×10^4	0.32
	J8	8.1×10^4	1.6×10^4	0.20
	SS	4.9×10^4	2.5×10^2	5.1×10^{-3}
	J	2.7×10^7	9.8×10^6	0.36
	J4	3.7×10^6	9.7×10^5	0.26
	J8	4.0×10^5	$<1.0 \times 10^2$	
	SS	3.4×10^5	$<1.0 \times 10^2$	
*ts*72	H	5.1×10^5	2.1×10^5	0.41
	H1	6.3×10^5	3.0×10^5	0.48
	SS	1.5×10^2	7	4.6×10^{-2}

[a] Marker rescue was performed by the two-step procedure. Individual experiments are shown for *ts*13 because of the variability of the efficiency of rescue with this mutant as well as the occasional occurrence of a low proportion of wild-type plaques in the control cultures that received salmon sperm (SS) DNA. The DNAs used are depicted in Fig. 1.

mRNA. Biochemical evidence that the RNA polymerase activity of *ts*13 and *ts*72 is altered will be presented elsewhere (M. Ensinger, manuscript in preparation).

The results presented in this paper demonstrate the power of using fine-structure marker rescue combined with transcriptional and translational mapping data as a means of characterizing mutants of a virus as large and complex as vaccinia virus. Thus, the sites of the *ts* mutations of the eight mutants described here have been located to regions of the viral genome that encode one or at most two polypeptides. Furthermore, the narrow limits defined for each mutation (in one case, 335 base pairs) by the marker rescue experiments will facilitate identification of the precise site of these mutations by sequence analysis. Detailed biochemical analysis of the phenotypic defects of these mutants will allow correlation of viral genes, polypeptide products, and their functions.

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LITERATURE CITED

1. **Bajszár, G., R. Wittek, J. P. Weir, and B. Moss.** 1983. Vaccinia virus thymidine kinase and neighboring genes: mRNAs and polypeptides of wild-type virus and putative nonsense mutants. J. Virol. **45**:62–72.
2. **Chernos, V. I., E. F. Belanov, and N. N. Vasilieva.** 1978. Temperature-sensitive mutants of vaccinia virus. I. Isolation and preliminary characterization. Acta Virol. **22**:81–90.
3. **Condit, R. C., and A. Motyczka.** 1981. Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. Virology **113**:224–241.
4. **Condit, R. C., A. Motyczka, and G. Spizz.** 1983. Isolation, characterization, and physical mapping of temperature-sensitive mutants of vaccinia virus. Virology **128**:429–443.
5. **Dales, S., V. Milovanovitch, B. G. T. Pogo, S. B. Weintraub, T. Huima, S. Wilton, and G. McFadden.** 1978. Biogenesis of vaccinia: isolation of conditional lethal mutants and electron microscopic characterization of their phenotypically expressed defects. Virology **84**:403–428.
6. **Drillien, R., D. Spehner, and A. Kirn.** 1982. Complementation and genetic linkage between vaccinia virus temperature-sensitive mutants. Virology **119**:372–381.
7. **Ensinger, M. J.** 1982. Isolation and genetic characterization of temperature-sensitive mutants of vaccinia virus WR. J. Virol. **43**:778–790.
8. **Ensinger, M. J., and M. Rovinsky.** 1983. Marker rescue of temperature-sensitive mutations of vaccinia virus WR: correlation of genetic and physical maps. J. Virol. **48**:419–428.
9. **Hruby, D. E., R. A. Maki, D. B. Miller, and L. A. Ball.** 1983. Five structure analysis and nucleotide sequence of the vaccinia virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **80**: 3411–3415.
10. **Lake, J. R., and P. D. Cooper.** 1980. Synthesis of virus DNA and polypeptides by temperature-sensitive mutants of rabbitpox virus. J. Gen. Virol. **47**:243–259.
11. **Mahr, A., and B. E. Roberts.** 1984. Organization of six early transcripts synthesized from a vaccinia virus *Eco*RI DNA fragment. J. Virol. **49**:497–509.
12. **Mahr, A., and B. E. Roberts.** 1984. Arrangement of late RNAs transcribed from a 7.1-kilobase *Eco*RI vaccinia virus DNA fragment. J. Virol. **49**:510–520.
13. **Plucienniczak, A., E. Schroeder, G. Zettlmeissel, and R. E. Streeck.** 1985. Nucleotide sequence of a cluster of early and late genes in a conserved segment of the vaccinia virus genome. Nucleic Acids Res. **13**:985–998.
14. **Sambrook, J. F., B. L. Padgett, and J. K. N. Tomkins.** 1966. Conditional lethal mutants of rabbitpox virus. I. Isolation of host cell-dependent and temperature-dependent mutants. Virology **28**:592–599.
15. **Weir, J. P., and B. Moss.** 1983. Nucleotide sequence of the vaccinia virus thymidine kinase gene and the nature of spontaneous frameshift mutations. J. Virol. **46**:530–537.
16. **Weir, J. P., and B. Moss.** 1984. Regulation of expression and nucleotide sequence of a late vaccinia virus gene. J. Virol. **51**:662–669.