Molecular Genetic Analysis of Vaccinia Virus DNA Polymerase Mutants

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To further our understanding of the structure and function of the vaccinia virus DNA polymerase, we have performed fine genetic analysis of three mutants with lesions in the polymerase gene. By performing marker rescue analysis with DNA fragments of decreasing size, each lesion was localized to within 500 base pairs of DNA. The relevant regions of the mutant alleles were then cloned and subjected to DNA sequence analysis, which allowed the assignment of a single nucleotide and amino acid change to each mutant. As well as providing structure-function correlations germane to an understanding of polymerase activity, these data have provided insights into the frequency and possible mechanisms of viral homologous recombination.

Faithful DNA replication is a vital biological process. Nevertheless, our knowledge of the full complement of enzymes, binding proteins, *cis*-acting regulatory sites, and template structural elements which contribute to the replication process is still incomplete. Although procaryotic systems have been highly amenable to analysis, our knowledge of eucaryotic DNA replication has been limited by the paucity of systems which lend themselves to combined genetic, molecular, and biochemical approaches. Viral systems are often good models because of the haploid genetic content and the ease of synchronization and manipulation of the infectious cycle. Nevertheless, most eucaryotic DNA viruses replicate within the host nucleus and employ host and viral functions, features which have complicated genetic and biochemical analysis. Vaccinia virus, a prototypic example of the poxvirus family, is distinguished by replicating solely within the cytoplasm of the infected cell and by possessing an apparent genetic autonomy from host functions (for a review, see reference 19). The vaccinia virus genome is thought to encode all of the functions required for DNA replication. Genetic analysis has led to the identification of the virally encoded thymidine kinase (12, 33), DNA polymerase (7, 14, 15, 20, 29, 31), and two other essential replicative functions. These later genes are marked by two complementation groups of temperature-sensitive DNA⁻ mutants but have not yet been assigned a function (6, 8, 22). Other enzymes involved in viral DNA metabolism, such as a nuclease (16), topoisomerase (1, 10, 23, 24), and ribonucleotide reductase (25-27, 30), are also under study. In addition to identification of the components of a metabolic pathway, molecular genetic analysis can provide an understanding of the molecular architecture of an enzyme or regulatory protein. Mutations often mark key domains involved in substrate or cofactor binding, active site function, regulation, or interaction with associated proteins.

For these reasons, we have been involved in a thorough molecular genetic analysis of the vaccinia virus DNA polymerase. The polymerase is a monomer of 110,000 kilodaltons and possesses both polymerization and 3' - 5' exonuclease functions (4, 5). The transcriptional and sequence analysis of the gene encoding the polymerase has been published previously (7, 14, 15, 31), as has been the preliminary mapping

of the lesions of polymerase mutants. By a combination of marker rescue analysis and DNA sequencing, we can now report the mapping of three polymerase lesions to the exact nucleotide and discuss the resultant amino acid alterations. In addition, the detailed marker rescue analyses performed in these studies and the resultant comparison of recombination and DNA sequence analyses offer insights into the system of homologous recombination of vaccinia virus.

MATERIALS AND METHODS

Cells and virus. Mouse L cells and African green monkey BSC40 cells were obtained from R. Condit and were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. *ts42*, NG26, and PAA⁺Vg were graciously provided by P. Sridhar and R. Condit and have been previously described (6, 29). For virological studies of these mutants, 32 and 39.5°C were used as the permissive and nonpermissive temperatures, respectively.

DNA preparation and molecular cloning. The wild-type *Hind*III-E clone was kindly provided by B. Moss. The *Hind*III E fragments from the genomes of *ts42* and PAA⁺Vg were generously provided by P. Sridhar and R. Condit. Genomic DNA from NG26 was obtained by harvesting infected cultures upon manifestation of maximum cytopathic effect. The cells were lysed by hypotonic swelling; the nuclei were removed by centrifugation, and the viral cores were then collected by centrifugation. The cores were treated by digestion with proteinase K-sodium dodecyl sulfate, and subsequently by extraction with phenol-chloroform (isoamyl) and with chloroform(isoamyl); the viral DNA was retrieved by ethanol precipitation. All restriction enzyme digestions were performed according to the instructions of the manufacturer. Addition of oligonucleotide linkers to DNA fragments was performed by standard ligation after filling in the staggered ends generated by restriction digests with the Klenow fragment of DNA polymerase I. DNA fragments were purified from agarose by glass powder purification (32). Purified fragments were subcloned into pBR322, pUC8, or the X18 derivative of pBR322 (B. Seed, personal communication) by standard procedures. Plasmids were grown in *Escherichia coli* HB 101 cells under appropriate antibiotic selection and were purified by the alkaline lysis procedure (13).

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FIG. 1. Identification of subclones within the DNA polymerase gene and their activity in marker rescue experiments. The position of the mRNA for the DNA polymerase gene (~~~~~) within the right half of the genomic *Hind*III E fragment is illustrated at the top. The 2,300 bp *Cla*I fragment previously shown to successfully marker rescue all of the mutant lesions is shown, and below this the positions of the subclones of the *Cla*I fragment generated for this report are indicated. DNA fragments which rescued all of the mutants (—), those which rescued *ts*42 (■), those which rescued NG26 (●), and those which did not rescue any of the mutants (□) are indicated. DNA clones are named by their delimiting restriction sites (from left to right with respect to the vaccinia virus genome) and by their sizes in base pairs. Restriction enzymes are abbreviated as follows: A, *Ava*II; B, *Bam*HI; C, *Cla*I; H, *Hind*III at the borders of *Hind*III E and *Hpa*II below; M, *Mlu*I; X, *Xba*I. The arrows at the bottom of the figure represent the sequencing strategy for the wild-type *Xba*I 1300 fragment and the appropriate regions of the mutant genes. Each arrow represents the sequencing of both DNA strands from a given end-labeled restriction site.

Marker rescue. Confluent monolayers of BSC40 cells (in 60-mm tissue culture dishes) were infected with the virus indicated at a multiplicity of infection of 0.03 and were maintained at the permissive temperature for 4 h (except where indicated in Table 1 for infection with wild-type virus). Linearized plasmid DNA (10 µg) was then added in the form of a calcium-phosphate precipitate; the cells were shifted to the nonpermissive temperature and maintained for 3 days (except where indicated in Table 1 for infection with wild-type virus). Cells and medium were then frozen, thawed, scraped from the dishes, and refrozen; the preparations were then sonicated for two 15-s bursts and analyzed by plaque assays performed both at the permissive and nonpermissive temperature. Titers of serial dilutions of the virus preparation were determined on BSC40 monolayers; the plates were fixed and stained with crystal violet after 48 h of incubation at 32, 37, or 39.5°C.

DNA sequencing. Plasmids were digested with the appropriate restriction enzyme and then either labeled at the 3' terminus with the Klenow fragment of DNA polymerase I and the appropriate [α - 32 P]dNTP or at the 5' terminus with polynucleotide kinase after dephosphorylation with calf intestinal alkaline phosphatase. Radiolabeled DNA was then subdigested to yield a DNA fragment labeled at a single terminus; fragments were purified by glass powder purification after agarose gel electrophoresis (32). Labeled fragments were sequenced by the Maxam-Gilbert chemical degradation procedure (18). For all data discussed in the

text, the sequence was obtained for both strands. Sequences were analyzed on an IBM PC computer by using the IBI DNA sequence software.

RESULTS

Our main goal was the determination of the lesions in several DNA polymerase mutants of vaccinia virus. *ts*42 is a temperature-sensitive (*ts*) mutant (6); NG26 is a double mutant with a temperature-sensitive lesion and a mutation rendering the virus resistant to the drug phosphonoacetic acid (PAA) (29). PAA is thought to be a competitive inhibitor of the pyrophosphate exchange site of the polymerase. The two mutations in NG26 were shown to be closely linked (29); by selection of a partial revertant which maintained PAA^r but was no longer temperature-sensitive, the virus PAA^rVg was generated (29). *ts*42 and NG26 thus provided us with two lesions which rendered the virus temperature sensitive and one which rendered it PAA^r. Marker rescue analysis with DNA subclones of decreasing size was performed to localize the lesions within a small (400- to 700-base-pair [bp]) region of the polymerase gene. In these experiments, cells were infected with the mutant virus, transfected with appropriate DNA fragments, and incubated for several days at the nonpermissive temperature. The transfected DNA is known to undergo homologous recombination with the viral genomes, and recombination events which generate wild-type virus lead to a spread of temperature-insensitive virus within

TABLE 1. Fine mapping of the *ts42* and NG26 lesions by marker rescue with subfragments of the DNA polymerase gene^a

DNA fragment	Titration of 39.5°C yield (PFU/ml) for:					Titration of wild-type vaccinia virus yield (PFU/ml) ^b	
	<i>ts42</i>		NG26			No PAA	PAA ^c
	32°C	39.5°C	32°C	39.5°C	39.5°C + PAA ^c		
None	2.7×10^3	$<10^1$	1.5×10^3 1.4×10^{3d}	$<10^1$	$<10^1$	2.0×10^8	1.0×10^3
1. <i>Cla</i> I 2300	3.5×10^6	2.5×10^6	2.0×10^6	2.0×10^6	2.0×10^5		
2. <i>Xba</i> I 1300	5.0×10^6	3.9×10^6	6.8×10^5	5.9×10^5	5.0×10^4		
3. <i>Xba</i> I- <i>Hpa</i> II 450	1.0×10^6	6.0×10^5					
4. <i>Hpa</i> II 850	2.0×10^3	$<10^1$					
5. <i>Xba</i> I- <i>Ava</i> II 600 ^e	1.2×10^4	1.1×10^4	2.0×10^3	$<10^1$	$<10^1$		
6. <i>Ava</i> II- <i>Xba</i> I 700 ^e	2.8×10^3	$<10^1$	8.0×10^4	5.2×10^4	3.5×10^3		
7. <i>Xba</i> I- <i>Mlu</i> I 1000 ^f			4.5×10^5	3.0×10^5	5.6×10^4		
8. <i>Mlu</i> I- <i>Xba</i> I 300 ^f			1.4×10^3	$<10^1$	$<10^1$		
9. PAA ⁺ <i>Xba</i> I 1300						1.1×10^8	2.7×10^5
10. <i>ts42 Xba</i> I- <i>Ava</i> I 600 ^g			$<10^1$	$<10^1$			

^a Marker rescue was performed as described in Materials and Methods. DNA fragments are named according to their delimiting restriction sites from left to right with respect to the vaccinia virus genome. Their relative positions are shown in Fig. 1.

^b These cultures were infected with wild-type vaccinia virus by using the standard marker rescue protocol, and where indicated were transfected with molecular clones of subfragments of the PAA⁺ DNA polymerase allele. PAA was added to 200 µg/ml at 15 h postinfection, and cultures were maintained for 48 h postinfection.

^c PAA (200 µg/ml) was included throughout the plaque assay.

^d Titer obtained in the presence of PAA (200 µg/ml).

^e The data for viral yields following infection with *ts42* and transfection with these two DNA fragments were obtained from a separate experiment in which the yield of virus from the *ts42* infection in the absence of added DNA was 4×10^3 PFU/ml when titer was determined at 32°C and $<10^1$ PFU/ml when titer was determined at 39.5°C.

^f These figures were obtained in a separate experiment in which the yield of virus released from NG26 infection in the absence of added DNA was 5×10^2 PFU/ml when titer was determined at 32°C and $<10^1$ PFU/ml when titer was determined at 39.5°C.

^g These figures were obtained in a separate experiment in which the yield of virus from the NG26 infection in the absence of transfected DNA was $<10^1$ PFU/ml when titer was determined at 32°C or 39.5°C. The yield of infection accompanied by transfection with *Ava*II *Xba*I 700 was 1.6×10^5 when titer was determined at 32°C and 1.7×10^5 when titer was determined at 39.5°C.

the culture. The same approach is applicable to the mapping of drug resistance. The approach of fine mapping was chosen to delimit an essential region suitable for sequencing. Sequence analysis of large regions of the various DNA polymerase alleles might uncover neutral polymorphisms generated during viral passage, which would complicate identification of the important mutation. By limiting sequence analysis to a small essential region, the chance of detecting irrelevant DNA changes was significantly reduced.

Marker rescue analysis of polymerase mutants. Our previous studies of the polymerase mutants had localized their lesions to a central 2.3-kilobase *Cla*I fragment within the gene (31). For the current work, a restriction map of the polymerase gene was developed, and several subclones were generated from the central region of the gene. These are indicated schematically in Fig. 1. Subclones of the wild-type gene were used to rescue *ts42* and NG26 to the temperature-insensitive phenotype. Representative data are shown in Table 1. Both temperature-sensitive lesions were localized within a 1,300-bp *Xba*I fragment. After subdivision of this fragment with *Hpa*II and *Ava*II, the lesion in *ts42* was mapped within a 450-bp *Xba*I-*Hpa*II fragment and the *ts* lesion of NG26 was mapped within 400 bp representing the overlap of a 700-bp *Ava*II-*Xba*I fragment and a 1,000-bp *Xba*I-*Mlu*I fragment. Because PAA strongly inhibits successful marker rescue (P. Sridhar and R. Condit, personal communication; E. Evans and P. Traktman, unpublished data), mapping of the PAA⁺ allele required a modified approach. As shown in the rightmost panel of Table 1, the ability of fragments of the PAA⁺ DNA polymerase allele to rescue wild-type virus to the PAA⁺ phenotype was tested. To bypass PAA inhibition of marker rescue, the first 15 h of infection-transfection were performed in the absence of selective pressure; PAA was added for the subsequent

incubation. The 2,300-bp *Cla*I fragment (data not shown) and the 1,300-bp *Xba*I fragment (Table 1) of the PAA⁺ allele could transfer drug resistance. However, the low frequency of recombination obtained with smaller DNA fragments under these nonselective conditions and the severe cytopathic effect induced by the high-multiplicity infections required to detect rare PAA⁺ recombinants prevented finer mapping of the PAA⁺ lesion with this approach. Our confidence that the *ts* and PAA⁺ lesions in NG26 were closely linked (0.065% recombination frequency [29]) led us to take another, more indirect approach. After selection for the rescue of NG26 to the temperature-insensitive phenotype by recombination with wild-type polymerase DNA as described above, the concomitant loss of PAA⁺ was also monitored. As seen in Table 1, each of the fragments which rescued NG26 to the temperature-insensitive phenotype (lines 1, 2, 7, and 8) restored sensitivity to PAA in approximately 82 to 95% of the recombinants. Thus, the PAA⁺ lesion falls within the same 400-bp region as does the *ts* lesion.

Sequence analysis of wild-type and mutant alleles. The sequences were obtained for the wild-type 1,300-bp *Xba*I fragment within which all of the mutants map as well as for the relevant segments of the *ts42*, NG26, and PAA⁺Vg alleles. The sequencing strategy for the wild-type (and mutant DNA) is shown schematically at the bottom of Fig. 1. In all cases, sequences were obtained for both strands of the DNA and were further confirmed by extensive overlap provided by the sequencing strategy. The wild-type sequence is shown in Fig. 2; the mutations are illustrated above the wild-type sequence and are highlighted in boxes. All three mutations are single G-to-A transitions with a resultant change in a single amino acid. The PAA⁺ mutation in NG26 and PAA⁺Vg results in a substitution of an *asp* residue for a *gly* residue and also creates a novel *Cla*I site.

FIG. 2. DNA sequence of the wild-type *Xba* 1300 fragment and identification of the lesions in *ts42* and NG26. The position of the *Xba* 1300 fragment relative to the genomic *Hind*III E fragment and the DNA polymerase mRNA is shown. The sequence corresponds to amino acids 251 to 673 of the complete DNA polymerase (7). The DNA sequence was determined by the strategy indicated in Fig. 1 as described in Materials and Methods. Both strands were sequenced completely. The only changes observed in the mutant alleles are indicated above the wild-type sequence and highlighted in boxes. Box a represents the lesion conferring PAA^r in NG26 and PAA^rVg. Box b represents the lesion causing temperature sensitivity in NG26. Box c represents the lesion conferring temperature sensitivity in *ts42*.

The *ts* lesion in NG26 maps 60 bp from the PAA^r lesion and also results in the substitution of an *asp* residue for a *gly* residue. The *ts* lesion in *ts42* results in the substitution of a *lys* residue for a *glu* residue.

DISCUSSION

To gain insight into the structure and function of the vaccinia virus DNA polymerase, we have conducted a molecular genetic analysis of one drug-resistant and two temperature-sensitive mutants with lesions within the polymerase gene. The lesions were mapped to within 500 bp by performing sequential marker rescue experiments with gene fragments of decreasing size. The marker rescue, mediated by homologous recombination between viral genomes introduced by infection and transfected DNA fragments, remained quite efficient even with clones carrying homologous inserts of a mere 450 bp. This finding strongly suggests that the virus, replicating solely within the cytoplasm, possesses a highly efficient recombination system worthy of genetic and biochemical analysis. The success of the fine mapping of

the lesions by genetic analysis made the comparison of the mutant alleles with the wild-type gene by DNA sequencing quite feasible. A 1,270-bp fragment of the wild-type gene (a DNA region which was sufficient to rescue all of the mutants to the wild-type phenotype) was sequenced; approximately 500 to 800 bp from each mutant allele was sequenced as well. While this work was in progress, the sequence of the entire polymerase gene was determined and published by other investigators (7; see reference 3 for correction). The sequence included in this report corresponds to amino acids 251 to 673 of the 981-amino-acid DNA polymerase. With the exception of a single base, the sequences are identical. (The C at position 62 of our sequence was reported as a T by Earl et al. [their position 1,308], with the resultant prediction of a Tyr where we predict a His [amino acid 271]. Our confidence in our sequence determination was strengthened by our observation of this sequence in the wild-type genomic clone obtained from B. Moss as well as in the NG26 and the PAA^rVg alleles. After consultation with the original authors to confirm their finding [7], it seems likely that the variant

sequence they observed arose during bacterial passage of plasmid DNA.)

A single-nucleotide change (with an accompanying amino acid change) was detected for each mutant. All three of the lesions we investigated were shown to be single G-to-A transitions. This chemical change is consistent with the use of nitrosoguanidine during their isolation. Interestingly, no additional polymorphisms were detected by our sequence analysis, indicating that the vaccinia virus DNA is genetically quite stable, and suggesting that the polymerase has a high degree of fidelity. The PAA^r lesion of NG26, identified as the substitution of an Asp for a Gly (at amino acid 347 of the complete DNA polymerase), is identical with the change found in another PAA^r mutant by Earl et al. (7). This is striking because the mutants were independently isolated in different laboratories. The repeated association of PAA^r with this change suggests that the alteration may pinpoint the site of PAA binding or inhibition. The *ts* lesion in NG26 also results from the substitution of an Asp for a Gly (amino acid residue 367 of the enzyme); this substitution of an acidic for an uncharged amino acid falls within a stretch of ten residues which are either uncharged or hydrophobic. The *ts* lesion in *ts42* maps in a different region of the gene (amino acid 586) and results from a substitution of a Lys for a Glu (amino acid 586 within the DNA polymerase). The latter represents a drastic charge alteration and may define an important region for catalytic activity, regulation, or proper protein folding. It is interesting to note that the two residues whose alteration results in the temperature-sensitive phenotype are conserved in the recently sequenced fowlpox DNA polymerase (3). Recent comparisons of the herpes simplex virus, adenovirus, Epstein-Barr virus, yeast, human, various procaryotic, and vaccinia virus polymerases have identified several conserved regions of strong homology (2, 7, 17, 21, 34). Some of these conserved domains lie within the 1,270 bp studied in this report, although none of the lesions we have identified affect the most highly conserved residues. Alterations of essential binding sites might inactivate the enzyme and be unconditionally lethal. However, the striking homologies observed across significant evolutionary distance suggest that detailed analysis of the vaccinia virus enzyme will provide insight into the general workings of DNA polymerases. Analysis of drug-resistant mutants of the herpes simplex virus DNA polymerase has facilitated the identification of important binding domains within the enzyme (11). Toward this end, molecular genetic analyses of aphidicolin-resistant and cytosine arabinoside-resistant mutants of vaccinia virus are also under way in our laboratory (J. Taddie and P. Traktman, unpublished results).

The nucleotide assignment for these lesions provides interesting insights into the efficiency and mechanism of viral recombination. Recombination between NG26-*ts* and *ts42* to yield temperature-insensitive virus was previously shown to occur with a frequency of 1.9% (29). We can now say that this recombination frequency occurs between lesions located 656 bp apart. The recombination frequency between the NG26 PAA^r and *ts* lesions was shown to be 0.065% (29), and we can now show that this represents a distance of 60 nucleotides. The correlation of recombination frequency with accurate mapping distance may help in future estimates of the distance between intragenic genetic markers, although these data suggest that recombination may not increase in a precisely linear fashion with increasing distance between markers. Sridhar and Condit, in their original presentation and analyses of these polymerase mutants, made use of recombination frequencies obtained from two- and three-

factor crosses to order the three markers as follows: NG26-*ts* - NG26-PAA^r - *ts42* (29). This conclusion was reached by comparing the frequency of obtaining temperature-insensitive, PAA^r viruses from NG26-*ts42* coinfections with the previously determined recombination frequencies between the two *ts* lesions and the two NG26 lesions. Our sequence analysis, however, clearly demonstrates that the order is NG26-PAA^r - NG26-*ts* - *ts42*. (To address the formal possibility that the recombination frequencies they observed reflected that viruses containing the *ts* lesions of both *ts42* and NG26 could be phenotypically temperature-insensitive, we attempted to rescue NG26 to the temperature-insensitive phenotype with a fragment from the *ts42* genome which contains the *ts* lesion. As shown in line 10 of Table 1, no temperature-insensitive progeny were generated.) Thus, a recombinational pathway which formally entails two crossover events occurred with the expected frequency for a single crossover between the two closest markers. A reasonable conclusion is that once DNA synapsis occurs and recombination is initiated, the number of crossovers may be insignificant and the final recombination frequency may be determined solely by the distance between the closest markers. Alternatively, the possibility that homologous recombination in vaccinia virus is mediated by gene conversion events must be investigated.

Table 1 provides additional evidence that multiple crossover events occur with high frequency during vaccinia virus infection. The middle panel of the table presents data regarding the unselected loss or maintenance of the PAA^r marker during selection for acquisition of the temperature-insensitive marker. Although the two markers are separated by only 60 bp, 5 to 10% of the temperature-insensitive recombinants do not acquire the closely linked PAA sensitivity. If we can demonstrate that PAA^r virus possesses no growth advantage, then these data suggest that recombination between these very close markers is surprisingly high. Moreover, the efficiency of the linearized *Xba*-*Mlu* fragment (shown in line 7 of Table 1) at rescuing NG26 to the temperature-insensitive phenotype (although the *ts*⁺ marker is flanked by only 119 bp of upstream vaccinia virus sequences) suggests that recombination between close markers is frequent.

The data presented in this report represent the beginning of a structure-function analysis of the vaccinia virus DNA polymerase. Identification of key areas within the enzyme has encouraged us to initiate site-directed mutagenesis (35) of the polymerase in regions which appear important by virtue of striking sequence features or homology to other DNA polymerases (2, 7, 17, 21, 34). The technology for subsequent reinsertion of altered alleles into the viral genome by marker rescue, even in the absence of a selectable phenotype, has recently been developed (9). We have also generated an antibody to the DNA polymerase (28) which will facilitate analysis of polymerase expression in vivo. The continued correlation of the position and nature of the amino acid change, the properties of the mutant enzyme, and the phenotype of the mutant virus should provide valuable insights into the structure and function of the DNA polymerase.

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