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Identification of Temperature-Sensitive Mutants of Vaccinia Virus That Are Defective in Conversion of Concatemeric Replicative Intermediates to the Mature Linear DNA Genome

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Pulsed-field gel electrophoresis was used to screen temperature-sensitive mutants of vaccinia virus for the ability to convert replicated viral DNA into mature linear 185-kilobase hairpin-terminated genomes. Of 30 mutually noncomplementing mutants tested, 5 displayed a temperature-sensitive defect in the resolution of the telomere fusion configuration within concatemeric replicative intermediates, resulting in a failure to convert such intermediates to the linear monomeric genome. Adjacent genomic units in the concatemeric arrays generated in these mutants were arranged in both tandem and inverted orientations. The observation that four of the five mutants had a severe general defect in the synthesis of the late class of viral proteins suggests that at least one late protein is directly required to resolve the telomere fusion intermediate to hairpin termini. The identification of such telomere resolution proteins should be facilitated by genetic and molecular characterization of resolution-defective mutants, such as C63, in which late protein synthesis is not severely affected.

The poxvirus family comprises a group of eucaryotic DNA viruses which characteristically replicate in a highly autonomous fashion in the cytoplasm of infected cells. Members of this family encode all or nearly all factors required for the replication of their large DNA genomes (37, 45), a property which makes them choice candidates to identify the corresponding genes and perform the genetic and molecular studies so vital to understand the mechanisms of biological phenomena such as DNA replication and recombination. Vaccinia virus, the prototype member of this family, has a 185-kilobase (kb) linear double-stranded DNA genome which includes a 10-kb terminal inverted repeat that is terminally crosslinked by an A+T-rich incompletely base-paired hairpin structure. The terminal hairpin exists in two isomeric configurations (flip and flop), which are inverted and complementary in sequence (reviewed in reference 31). This genome structure is conserved among poxviruses.

Mechanisms used by poxviruses to replicate their DNA are still ill defined. DNA replication takes place within specialized structures called "factories" or "virosomes." Initiation of DNA replication has been examined by electron microscopy and in vivo labeling of early replicating DNA (17, 35, 36). The results suggest the existence of a replication origin at or near the hairpin terminus. The existence of a specific origin sequence could not be confirmed by the in vivo plasmid replication assay (21), since plasmids transfected into poxvirus-infected cells replicate efficiently irrespective of the presence of poxvirus DNA sequences (11, 28). The mechanism by which transfected plasmid DNA is replicated is not understood. However, the observation that extensive recombination between and within exogenous plasmid DNA coincides with its replication (13, 18) may indicate that a replicative recombination pathway similar to that observed in T4 bacteriophage (for a review, see reference 30) is operative in poxviruses.

Newly replicated poxvirus DNA exists as high-molecular-weight aggregates (9), which have been postulated to consist of transient circular intermediates (1, 27) or concatemeric arrays (32, 33), and which are converted into mature hairpin-terminated genomes. Replication of the hairpin termini generates replicative intermediate forms that exist as inverted repeat configurations in which the two isomeric (flip and flop) hairpin strands are base-paired (13, 28, 32, 33). This "telomere fusion" configuration, which connects two adjacent terminal inverted repeats within replicative intermediate structures, harbors the *cis*-acting DNA sequence that facilitates their conversion to hairpin termini (12, 13, 28).

Genetic analysis of the vaccinia virus infective cycle has been initiated in several laboratories through the isolation of temperature-sensitive mutants (6–8, 10, 14, 15), many of which have been screened for defective DNA replication by monitoring their ability to incorporate [^{3}H]thymidine into viral DNA. This method has permitted identification of three DNA-negative complementation groups, whose wild-type genes are required for viral DNA synthesis (7, 8).

This article reports a rapid and sensitive method of screening mutants with replication defects that would have gone undetected with an in vivo labeling protocol. Specifically, the detection of altered conformational states of replicated viral DNA requires a method that allows facile distinction between replicative intermediates and the 185-kb linear viral genomes. Pulsed-field gel electrophoresis (PFGE) and field inversion gel electrophoresis are sensitive size-dependent separation procedures for linear DNA molecules up to about 10,000 and 2,000 kb, respectively (4, 5, 39, 40), and allow separation of large circular DNA molecules that cannot enter the gel under conventional electrophoresis conditions (26). I show the use of PFGE as a tool to rapidly detect and characterize replication defects among temperature-sensitive mutants of vaccinia virus and show the existence of several classes of mutants that are incapable of converting concatemeric replicative intermediates into the mature linear 185-kb genome. The data suggest that this conversion event is dependent on one or more late viral gene products.

MATERIALS AND METHODS

Cells and virus. Continuous lines of African green monkey kidney cells (BGMK or BSC40 cells) were grown in monolayer cultures in Dulbecco modified Eagle (DME) medium supplemented with 5% fetal calf serum. Wild-type vaccinia

virus WR and mutants (*ts* C21, C37, C40, C42, C45, C46, C49, C52, C53, C56, C57, C60, C63, C5, C6, C7, C8, C11, C13, C17, C50, C51, C25, C22, C33, C16, C1, C30, C18, and C12) representing 30 complementation groups were obtained from R. Condit (7, 8, 43). In this report, these mutants are referred to with the prefix C. Virus stocks were passaged at low multiplicity (0.05 to 0.1). Crude virus preparations were routinely obtained by three cycles of freeze-thawing of 48-h-infected BSC40 cells. Propagation, purification, and titration of wild-type virus and temperature-sensitive mutants were done as described before (7, 8).

PFGE. Monolayers of BSC40 cells, infected at a multiplicity of 1 PFU per cell, were harvested by scraping the infected cells into the culture medium and pelleting cells at 700 × *g*, followed by suspension in a small volume (the amount depending on desired cell density, usually 10^7 cells per ml) of phosphate-buffered saline (PBS) with 40 mM EDTA. This cell suspension was placed at 40°C for at least 10 min before being mixed with an equal volume of 1% low-melting-point agarose (Bio-Rad Laboratories) containing 0.125 M EDTA which had been warmed at 43°C.

The cell-agarose mixture was pipetted into plastic molds (2 by 5 by 10 mm) and allowed to solidify. The resulting agarose blocks were treated for 24 h in a solution containing 1% Sarkosyl, 100 μg of proteinase K per ml, 0.18 M EDTA, and 10 mM Tris chloride (pH 7.5), equilibrated with agitation at 4°C against two changes of electrophoresis running buffer (TBE; 0.1 M TBE is 0.1 M Tris, 0.1 M boric acid, 2 mM EDTA), after which they were stored in 0.1 M TBE at 4°C. Just prior to electrophoresis, agarose blocks (2 by 5 by 5 mm) were inserted in premolded wells and sealed with 0.5% low-melting-point agarose. PFGE was performed in the LKB 2015 Pulsaphor electrophoresis unit. Initial gels (1.5% agarose, 0.1 M TBE) were run with electrodes set for a dual nonhomogeneous field of electrophoresis (5). In later runs, homogeneous fields, obtained with the use of a hexagonal electrode system (LKB), ensured straight lanes rather than the bent lanes routinely obtained with nonhomogeneous fields. The latter electrode system facilitates homogeneous electrophoresis conditions with an angle of 110° between the alternating currents. Gels were run at 200 mA with pulse times as indicated, usually 25 to 100 s, and the electrophoresis buffer was held at 15°C by means of cooling coils and a circulating-water bath. With the hexagonal electrode system, DNA samples were allowed to enter the gel at 5 V/cm for 45 min to promote sharper bands, followed by pulsing at indicated intervals and durations.

Following electrophoresis, DNA was partially depurinated by 15 min of incubation in 0.25 N HCl, transferred to a nitrocellulose membrane by Southern blotting (42), and hybridized with a ^{32}P-labeled probe. Probes were prepared by the random hexanucleotide primer method (19) with [^{32}P]dCTP and [^{32}P]dATP (Amersham). Washed blots were exposed to Kodak X-Omat R film with a Cronex Lightning-Plus intensifier screen (Du Pont Co.) at −70°C.

Restriction enzyme digestion of high-molecular-weight DNA. To further characterize the nature of DNA visualized by PFGE, DNA from agarose blocks was run in 1.5% low-melting-point agarose–0.1 M TBE and stained briefly with 1.0 μg of ethidium bromide per ml, and individual bands were excised. The excised bands (1 to 1.5 mm thick) were first equilibrated at 0°C against two changes of TE (10 mM Tris chloride [pH 7.5], 0.1 mM EDTA) and then against two changes of restriction enzyme buffer (as recommended by the supplier), and finally digestion was allowed to take place for at least 8 h at 37°C for *Xho*I or 30°C for *Sma*I in the presence of 0.2 U of restriction enzyme per μl. Agarose plugs thus treated were placed in wells and electrophoresed under conventional or PFGE conditions of electrophoresis. The *Xho*I-digested DNA was electrophoresed in 0.7% agarose for 18 h at 1.25 V/cm, and the *Sma*I-digested DNA was electrophoresed in 1.5% agarose at 200 mA, with 30-s pulses for 16 h and 8-s pulses for 8 h. The gels were then blotted and probed with plasmid pVT1, which contains a 1.4-kb *Sau*3A fragment from the region of tandem repeats near the termini of the vaccinia virus genome.

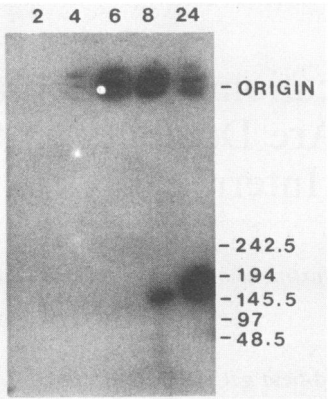

FIG. 1. PFGE analysis of replicating and replicated DNA from wild-type vaccinia virus. Monolayers of BSC40 cells were infected at a multiplicity of 5 with wild-type vaccinia virus strain WR, and at 2, 4, 6, 8, and 24 h after infection, infected cells were embedded in agarose blocks, treated with Sarkosyl and proteinase K (see Materials and Methods section), and subjected to PFGE analysis. DNA was electrophoresed for 48 h in 200-mA nonhomogeneous fields which alternated at 25-s intervals, allowing separation of linear DNA molecules up to about 400 kb. The DNA was then transferred to a nitrocellulose membrane and hybridized with plasmid pAB208 (obtained from A. Ball), which contains a 4-kb DNA fragment of vaccinia virus. Bacteriophage lambda oligomers were used as size standards. Sizes are indicated in kilobase pairs.

Protein synthesis and SDS-PAGE. Techniques for [^{35}S]-methionine pulse-labeling of proteins within vaccinia virus-infected cells and for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography of labeled samples were basically as described by Condit and Motyczka (7). Briefly, monolayers of BSC40 cells were infected at a multiplicity of between 5 and 50, incubated at 32 or 40°C for various periods of time, washed twice with warmed PBS, and pulse-labeled for 15 min in warmed PBS containing 20 μCi of [^{35}S]methionine (1,100 Ci/mmol; Amersham) per ml at either 32 or 40°C. The proteins were then solubilized in electrophoresis sample buffer (22), boiled for 4 min, frozen at −20°C, and reboiled for 4 min prior to electrophoresis on 10% or 12.5% polyacrylamide–SDS slab gels, with the mini-Protean gel system from Bio-Rad. The gels were fixed and stained in 40% methanol–10% acetic acid–0.1% Coomassie blue R-250, dried under vacuum, and autoradiographed.

RESULTS

PFGE of wild-type vaccinia virus. The migration of replicating and replicated wild-type vaccinia virus DNA in pulsed-field gels was examined by analysis of viral DNA isolated at 2, 4, 6, 8, and 24 h postinfection (Fig. 1). The earliest detectable viral DNA, visible at 4 h postinfection, remained associated with the agarose matrix in the well, as

was the great majority of high-molecular-weight cellular DNA. The viral replicative intermediates in the "well" fraction became prominent at 6 h postinfection, at which time a small amount of a linear DNA molecule appeared, which migrated at 180 to 190 kb, as would be expected for the 185-kb linear vaccinia virus genome. This band will henceforth be referred to as the 185-kb monomer or mature genome. The monomer band became prominent at 8 h postinfection and reached a maximum level sometime between 8 and 24 h postinfection. At 24 h postinfection, very little replicating DNA (well fraction) remained. No significant differences between efficiencies of DNA replication at 32 and 40°C have been detected. The only consistent difference was the presence of a weak slower-migrating band, which was present at 32°C but absent at 40°C. This band represents a linear dimeric molecule (see next section).

PFGE of temperature-sensitive mutants. To monitor replication defects among a collection of 30 noncomplementing temperature-sensitive mutants, monolayers of BGMK cells were infected with individual mutants at a multiplicity of 0.5 and incubated at 40°C for 48 h; agarose blocks were prepared as usual and electrophoresed under the PFGE conditions described in the legend to Fig. 1. This analysis revealed four classes of mutants. First, the majority of mutants (21 of 30) behaved like the wild type (Fig. 1, 24 h postinfection). Second, mutants C42 and C17 did not generate newly replicated DNA at 40°C. These mutants were previously identified as DNA negative by failure to incorporate [^{3}H]thymidine. Third, mutants C25 and C50, when grown at 40°C, produced levels of the 185-kb monomer band which were reduced at least 10-fold. C25 has previously been identified as a DNA-negative mutant, whereas no DNA replication defect has been reported for C50. Fourth, five mutants (C21, C53, C63, C7, and C22) displayed, in addition to a weak monomer 185-kb band, significant quantities of slower-migrating DNA as well as a high proportion of DNA that was incapable of entering the gel.

Among the 30 mutants tested, 9 displayed either quantitative or qualitative defects in viral DNA replication. Three of these mutants are DNA negative. Interestingly, the remaining six mutants share a "defective late" or "abortive late" protein synthesis phenotype (8). Conversely, only a single defective late mutant, C56, had no apparent defect in viral DNA replication. The defective late class of mutants was characterized in more detail. Infected monolayers of BSC40 cells were incubated for 24 h at 32 or 40°C, after which DNA was prepared in agarose blocks and subjected to PFGE analysis under conditions that enable separation of linear DNA molecules up to about 1,000 kb. Both wild-type virus WR and mutant C56 replicated their genomes with equal efficiency into 185-kb monomers at either 32 or 40°C (Fig. 2 and 3; lanes 1, 2, 7, and 8). In contrast, mutant C50 (Fig. 2 and 3; lanes 11 and 12) consistently replicated about 10-fold less DNA at 40°C than at 32°C. Virtually all replicated DNA was present as an apparently mature linear monomer. This result appears to be in contradiction to a previous report, in which no reduction in the amount of DNA synthesis was detected when incorporation of [^{3}H]thymidine was measured. The discrepancy appears to be due to the fact that previous measurements were done at a multiplicity of 10 rather than 1. After increasing the multiplicity to 10 and 40 and analysis by PFGE, little or no reduction in the total amount of DNA synthesis was observed (not shown).

The phenotype of four of five mutants (C21, C53, C7, C22, and C63) with slowly and nonmigrating DNA species is illustrated in Fig. 2, 3, and 4. At 32°C, these five mutants

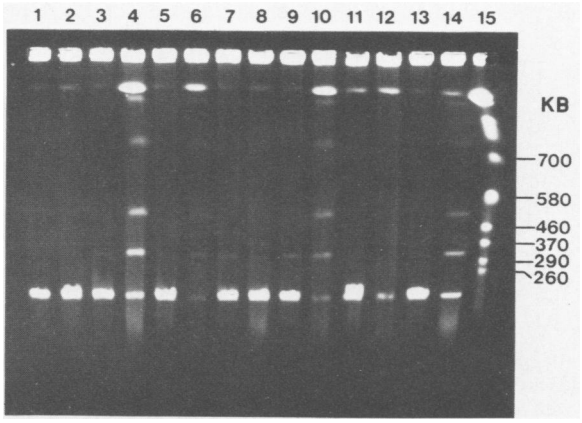

FIG. 2. PFGE analysis of DNA from temperature-sensitive mutants of vaccinia virus: ethidium bromide-stained gel. Monolayers of BSC40 cells were infected at a multiplicity of 1 with wild-type WR (lanes 1 and 2) and with mutants C21 (lanes 3 and 4), C53 (lanes 5 and 6), C56 (lanes 7 and 8), C7 (lanes 9 and 10), C50 (lanes 11 and 12), and C22 (lanes 13 and 14). Infected monolayers were incubated for 24 h at 32°C (odd-numbered lanes) or 40°C (even-numbered lanes), after which DNA was prepared and PFGE analysis was performed as described in the legend to Fig. 1, except that homogeneous fields were used and currents were alternated at 100-s intervals to allow separation of linear DNA molecules up to about 1,000 kb. To facilitate visualization of concatemer bands, 7.5×10^5 rather than 2.5×10^5 cells were used in lane 4. Yeast chromosomes were used as size standards (lane 15).

behaved like the wild type. In each case the great majority of replicated DNA resolved into the mature 185-kb monomer genome. However, at 40°C all five mutants displayed a characteristic pattern of DNA molecules which, in addition to a reduced amount of the 185-kb monomer, contained a ladder of slow-migrating DNA species as well as an abnormally high proportion of viral DNA that was unable to enter the gel matrix. This pattern of slow-migrating DNA molecules was not affected by changes in multiplicity of infection, at least in the range between 1 and 25. (Figure 4 shows the

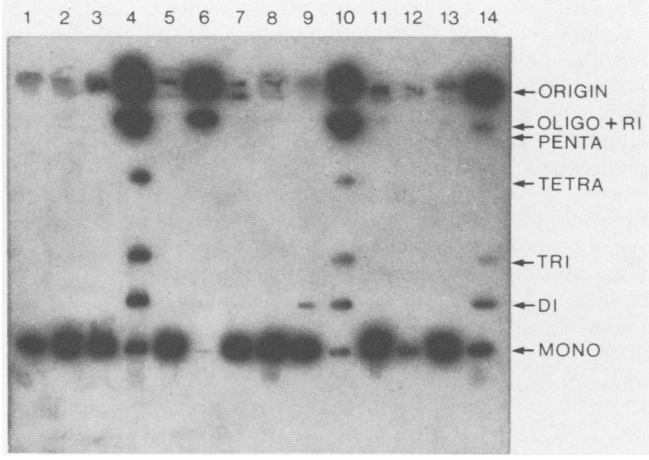

FIG. 3. PFGE analysis of DNA from temperature-sensitive mutants of vaccinia virus: Southern blot. The gel illustrated in Fig. 2 was blotted and hybridized with vaccinia virus DNA (Fig. 1). Members of the oligomeric series are indicated. The slowest-migrating band represents longer linear oligomers (oligo) and apparently nonlinear replicative-intermediate (RI) DNA (see text). See Fig. 2 legend for lane details.

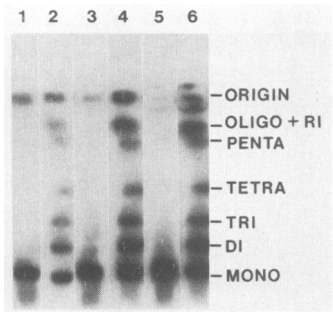

FIG. 4. PFGE analysis of DNA from C63-infected cells. Monolayers of BSC40 cells were infected at a multiplicity of 1 (lanes 1 and 2) 5 (lanes 3 and 4) or 25 (lanes 5 and 6). Infected monolayers were incubated for 24 h at 32°C (lanes 1, 3, and 5) or 40°C (lanes 2, 4, and 6), after which DNA was prepared and PFGE analysis was performed as described in the legend to Fig. 2. Members of the oligomeric series are as described in the legend to Fig. 3.

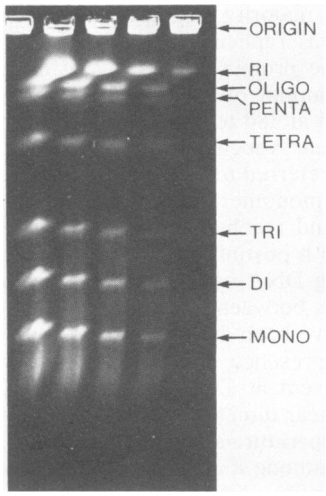

FIG. 5. Identification of linear and nonlinear concatemers from C21-infected cells. Monolayers of BSC40 cells were infected at a multiplicity of 1 and incubated at 40°C for 24 h, and DNA was prepared as described in Materials and Methods. Conditions of the first dimension of PFGE were as described in the legend to Fig. 2. The resulting concatemeric array of fragments generated by PFGE was exposed to conventional electrophoresis at a 55° angle to the first dimension. This was achieved by turning off the switching device used for PFGE. The four lanes contain DNA from 2×10^6, 1×10^6, 5×10^5, and 2.5×10^5 cells, respectively, from left to right. The gel was stained with ethidium bromide and photographed. Linear monomers (mono), oligomers (di, tri, tetra, penta, and oligo), and a nonlinear presumed replicative intermediate (RI) hybridized with vaccinia virus DNA (Southern blot not shown).

effect of multiplicity of infection on replication products of mutant C63.) That the relative proportions of slow-migrating DNA were invariant as a function of time after infection was demonstrated by time course analysis of each of these mutants. Whether analyzed at 6 to 8 h postinfection, when viral DNA first entered the gel, or at 24 or 48 h postinfection, no qualitative differences were detected. That the ladder of slow-migrating DNA molecules represented a series of linear oligomers of the 185-kb vaccinia virus genome was initially suggested by their apparent sizes on pulsed-field gels. Several lines of evidence confirm both the linear and oligomeric nature of these DNA species. First, during conventional gel electrophoresis, all linear DNA molecules above a certain molecular weight migrate at a constant identical rate through sieving by the gel matrix, and relaxed circular DNA molecules above a certain size fail to enter the gel matrix (26). When the ladder of DNA molecules generated by mutant C21 on a pulsed-field gel was subjected to conventional electrophoresis at an angle to the initial direction used during PFGE, it was found that all except the slowest-moving band migrated at identical rates (Fig. 5), as would be expected of linear DNA molecules in the observed size range. The slowest-moving band consisted of two components, a linear component capable of migrating under conventional electrophoresis conditions, and an apparently nonlinear component, called RI in Fig. 3 and 5, which failed to migrate under those conditions. Second, oligomeric forms of the linear genome of a poxvirus are expected to consist of multiple components of the unit genome which are linked by the inverted repeat "telomere fusion" configuration. When members of the oligomeric ladder from mutant C21 were excised and digested with the restriction enzyme *Xho*I, hairpin-terminated and telomere fusion fragments of 6.2 and 12.4 kb, respectively, were obtained in ratios expected for an oligomeric ladder, i.e., the fast-migrating monomer band of 185 kb contained only the 6.2-kb terminal hairpin fragments, whereas the slowest-migrating members of the oligomeric series harbored predominantly 12.4-kb telomere fusion fragments (Fig. 6). When the well fraction of nonmigrating DNA was similarly analyzed, the telomere fusion configuration again constituted the predominant component (Fig. 6C, lane 6). Third, digestion of the oligomers with the restriction enzyme *Sma*I, which cleaves the mature vaccinia virus DNA genome once, generating 35-kb and 150-kb fragments, resulted in the additional appearance of double-size (70 and 300 kb) and of genome-size (185 kb) digestion products (Fig. 7). The double-size molecules represent the inverted orientation and the genome-size molecules the tandem orientation of adjacent genomic units of the concatemers (Fig. 7B). The slower-migrating members of the oligomeric DNA molecules generated increased quantities of the 70-kb, 185-kb, and 300-kb fragments, and correspondingly reduced amounts of the terminal 35-kb and 150-kb fragments, as would be expected of an oligomeric series.

Protein synthesis: SDS-PAGE of temperature-sensitive mutants. The 30 mutants analyzed in this study have been tested previously for their ability to incorporate [^{3}H]thymidine into viral DNA and for the ability to synthesize both early proteins (before DNA replication) and late proteins (after the onset of DNA replication) (7, 8). On the basis of these experiments, it was shown that a class of DNA-positive mutants, representing seven complementation groups, displayed characteristically defective or abortive synthesis of late proteins. Upon examination of the 30 mutants by PFGE, I observed a nearly perfect correlation between defective DNA replication and late-protein synthesis. Thus, mutants with normal protein synthesis generated normal amounts of the mature 185-kb genome. Conversely, six of seven abortive late and defective late mutants produced either reduced amounts of DNA or a characteristic pattern of linear and nonlinear concatemeric DNA (Table 1). Defective late mutants are a highly heterogeneous class which includes mutants that exhibit a very extreme shut-down of late-protein synthesis and others with only slightly delayed or reduced protein synthesis (8). In an attempt to correlate the differences in the protein synthesis phenotype with defects at the DNA level, I analyzed protein synthesis in cells infected with each of the seven defective or abortive late mutants. Even though, as expected, all seven mutants displayed a

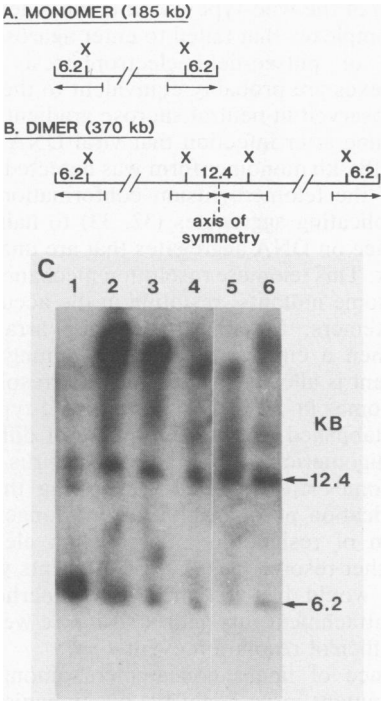

FIG. 6. Prevalence of the hairpin and telomere fusion conformations within members of the oligomeric series from C21-infected cells. The 185-kb monomer genome (A) and the 370-kb dimer (B) contain 10-kb terminal inverted repeats (indicated by arrows). *Xho*I (X) cleaves within these repeats to generate a 6.2-kb terminal hairpin fragment and a 12.4-kb telomere fusion fragment (see text). (C) Monolayers of BSC40 cells were infected with mutant C21, and the infected monolayers were incubated for 24 h at 40°C. The resulting oligomers were excised from the agarose gel, digested in situ with *Xho*I, and electrophoresed in 0.7% agarose gel under conventional electrophoresis conditions, as described in Materials and Methods. The gel was blotted and hybridized with a 1.4-kb vaccinia virus terminal DNA fragment. The 6.2-kb (hairpin) and 12.4-kb (telomere fusion) fragments are indicated by arrows. Lane 1, Monomer; lane 2, dimer; lane 3, trimer; lane 4, tetramer; lane 5, oligomer plus replicative intermediate (see Fig. 5); lane 6; origin or well fraction. The faint bands in lane 1 (at approx. 11 and 12 kb) represent partial digestion products, and the slow-migrating bands in lanes 2 to 6 probably represent undigested or partially digested high-molecular-weight DNA.

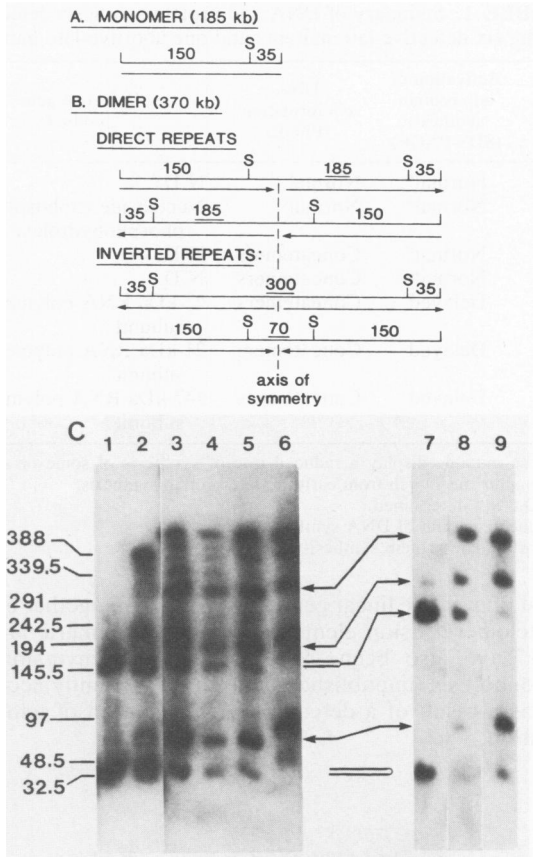

FIG. 7. Structure of high-molecular-weight concatemers from C21-infected cells. The 185-kb monomer (lanes 1 and 7), dimer (lane 2), trimer (lanes 3 and 8), tetramer (lane 4), oligomer plus replicative intermediate (lane 5), and nonmigrating well DNA (lanes 6 and 9) were excised from low-melting-point agarose as described in the legend to Fig. 6 and digested in situ with *Sma*I (S), and the resulting restriction fragments were analyzed by PFGE as described in Materials and Methods. *Sma*I cleaves the 185-kb monomer genome of vaccinia virus once to produce 35-kb and 150-kb fragments (A; C, lanes 1 and 7). *Sma*I digestion of the 370-kb dimer generates additional fragments of 70, 185, and 300 kb, each of which contains a telomere fusion element (B; C, lane 2). These telomere fusion fragments (indicated by arrows in panel C) were obtained from dimers, trimers, tetramers, oligomers plus replicative intermediates, and the nonmigrating well DNA. As expected, the proportion of the 35-kb and 150-kb hairpin-containing fragments (indicated by "hairpins" in panel C) decreases with increasing size of the oligomers. Note that lanes 1 to 6 represent incomplete digests and that the observed partial digestion products, e.g., two fragments in the 185- to 300-kb range, are compatible with the proposed oligomeric structures (B). Complete digests (C, lanes 7 to 9) show that only the two hairpin fragments were generated from the 185-kb monomer (C, lane 7) and that three additional (telomere fusion) fragments were generated from the oligomers (C, lane 8) and nonmigrating well DNA (C, lane 9). Sizes (in kilobase pairs) were determined from lambda oligomer standards.

defective or abortive late phenotype, the patterns of proteins on SDS-polyacrylamide gels permitted subdivision of these mutants on the basis of the kinetics of activation of late-protein synthesis at 40°C. Mutants C21, C53, and C7 displayed a characteristically much delayed onset of late-protein synthesis, which never approached the wild-type level. The remaining mutants were shown to initiate expression of at least the more highly expressed late proteins with wild-type kinetics. The apparently normal switch to late-protein synthesis between 2 and 5 h postinfection was then followed by a gradual decline (C50, C56, and C63) or an abrupt cessation (C22) of the synthesis of some or all late proteins. Figure 8 shows the synthesis of late proteins as early as 5 h after infection with wild-type WR or with mutants C50 and C63.

DISCUSSION

PFGE has been used successfully to screen temperature-sensitive mutants of vaccinia virus for defects during the termination phase of viral DNA replication. This technique was shown to be a sensitive and relatively rapid way to detect not only the mature viral 185-kb DNA genome, but also a series of linear concatemers and as yet uncharacterized nonlinear DNA structures. Characteristic concatemeric arrays were generated during replication of each of five noncomplementing mutants at the nonpermissive temperature. The linear concatemers in these arrays were shown to

TABLE 1. Summary of DNA and protein synthesis defects among six defective-late mutants and one abortive-late mutant

Mutant	Activation of late-protein synthesis (SDS-PAGE)	DNA conformation (PFGE)	Defective gene product
C56	Normal[a]	Normal	N.D.[b]
C50	Normal[a]	Normal[c]	Nucleoside triphosphate phosphohydrolase I
C22	Normal[d]	Concatemers	N.D.
C63	Normal[a]	Concatemers	N.D.
C7	Delayed	Concatemers	22-kDa RNA polymerase subunit
C21	Delayed	Concatemers	21-kDa RNA polymerase subunit?
C53	Delayed	Concatemers	147-kDa RNA polymerase subunit

[a] These mutants display a reduced rate of synthesis of some or all late proteins after the switch from early- to late-protein synthesis.
[b] N.D., Not determined.
[c] Reduced amount of DNA synthesis (see text).
[d] Abortive late-protein synthesis (34).

consist of multiple linear genome units linked together by the viral telomere fusion element. These concatemeric arrays, which have also been visualized by field inversion gel electrophoresis (unpublished data), have evidently accumulated as a result of a defect in the mechanism of telomere resolution.

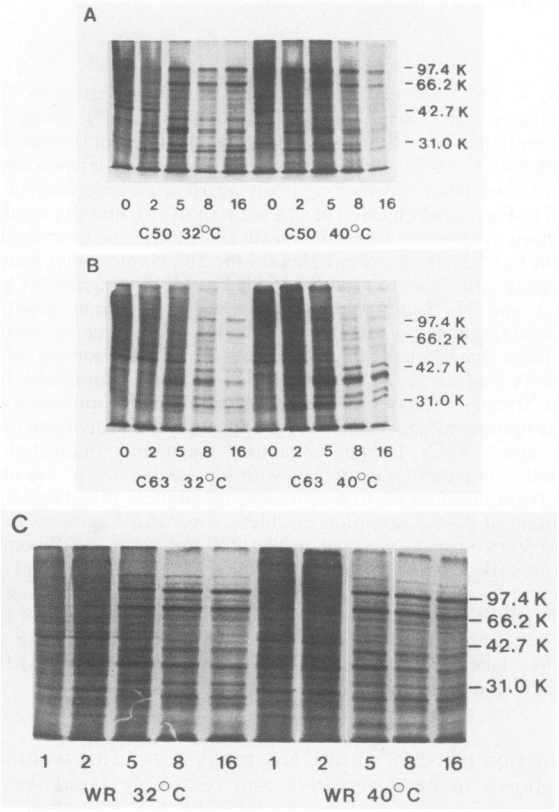

FIG. 8. Protein synthesis in mutants C50 (A), C63 (B), and wild-type strain WR (C). BSC40 cells were infected at a multiplicity of 20 and incubated for 0, 1, 2, 5, 8, or 16 h at 32 or 40°C, after which they were pulse-labeled with [^{35}S]methionine and samples were electrophoresed on 12.5% (A and B) or 10% (C) polyacrylamide–SDS gels. Molecular masses are indicated in kilodaltons.

Replication of the wild-type vaccinia virus genome generated DNA complexes that failed to enter agarose gels under conventional or pulsed-field electrophoresis conditions. These complexes are probably equivalent to the large DNA aggregates observed in neutral sucrose gradients (9, 16). At the earliest time after infection that viral DNA entered the gel, only the 185-kb monomer form was detected. Therefore, resolution of the telomere fusion conformation present in wild-type replicating aggregates (32, 33) to hairpin termini must take place on DNA aggregates that are unable to enter the gel matrix. This telomere resolution mechanism is clearly impaired in some mutants, resulting in the accumulation of linear concatemers. Linear concatemeric arrays are also generated when a circular plasmid containing a telomere fusion fragment is allowed to replicate and resolve to linear minichromosomes in cells infected with wild-type virus (13, 28). Once established, the relative ratios of different members of the oligomeric arrays of either poxvirus genomes or minichromosomes are invariant, suggesting that they are terminal replication products that are no longer substrates for resolution of resident telomere fusion elements. The failure to further resolve these fusion elements within linear concatemers would be explained if a superhelical DNA substrate or attachment to a matrix structure were a prerequisite to an efficient resolution event.

The presence of linear concatemers among the DNA products of mutant virus is always accompanied by a high proportion of apparently nonlinear replicative DNA, most of which never enters the gel matrix. The existence of long stretches of linear concatemeric DNA (300 kb and longer) within these DNA aggregates, demonstrated by digestion of such DNA with the restriction enzyme *Sma*I, supports the notion that the mutants are defective in the conversion of the telomere fusion elements to hairpin termini. The failure of these concatemeric DNA aggregates to enter agarose gel matrixes would be explained if they contain replicating DNA or consist of fully replicated DNA that is either circular, partially circular, or attached to a Sarkosyl–proteinase K-resistant matrix. In any case, inefficient resolution of the telomere fusion elements would release large linear concatemers from the DNA aggregates. The remaining nonlinear structures would fail to enter the gel or migrate slowly under PFGE conditions of electrophoresis. The observation that the intercalating drug ethidium bromide inhibits replication of a fast-sedimenting replicative intermediate suggests the existence of a circular or partially circular replicative intermediate (1). Circular structures could act as precursors to the replication of fast-sedimenting concatemeric replicative intermediates, which are then converted to hairpin-terminated linear DNA molecules by resolution of the hairpin fusion elements.

Digestion of the concatemers with the restriction endonuclease *Sma*I, which cleaves vaccinia virus DNA once, has demonstrated that both tandem and inverted orientations of adjacent units in the concatemeric arrays exist with about equal incidence. This observation is in contrast to the observed structure of replicating rabbit poxvirus DNA, in which the inverted orientation predominates (33). My results are compatible with the model proposed by Moyer and Graves (33), which postulates replication of linear DNA genomes into concatemers in which adjacent genome units are present in the inverted orientation. Subsequent recombination between the terminal inverted repeats of nonadjacent units promotes conversion to both tandem and inverted orientations. The defective resolution of concatemers in mutant virus would be expected to extend the time period

needed to facilitate such recombination events and thereby increase the proportion of tandem orientations.

The onset of viral DNA replication is a prerequisite for synthesis of late viral proteins. Consequently, DNA-negative mutants fail to produce late proteins. In addition, late viral protein synthesis is defective or abortive in several DNA-positive mutants (7, 8, 23). It has now been found that five of seven defective or abortive late mutants of vaccinia virus are unable to convert replicated concatemer intermediates to mature viral genomes. In agreement with this observation, the same mutants have recently been shown to be defective in the conversion of telomere fusion-containing plasmids to linear minichromosomes (29). In contrast, no DNA replication defects were observed among mutants that display normal protein synthesis. These data suggest that the resolution of concatemeric replicative intermediates requires the function of one or more late gene products. The accumulation of concatemers in mutant virus may be an indirect effect due to a general failure to synthesize sufficient quantities of late proteins or a direct effect of a defective concatemer resolution protein. An example of a mutant that appears to act in an indirect fashion is provided by the abortive late mutant C22, in which the failure to synthesize late proteins is caused by the rapid degradation of RNA (34). The RNA polymerase mutants C7, C51, and C53 represent another class of genes that may indirectly affect concatemer resolution. Mutants C7 and C53, which have lesions in the 22- and 147-kDa subunits of the virus-encoded RNA polymerase, respectively, combine a severe defect in protein synthesis (20, 44) and defective resolution of concatemeric replicative intermediates. Mutant C21, which is probably defective for the 21-kDa RNA polymerase subunit (41), has a similar phenotype. Conversely, both protein synthesis and DNA replication are normal in another mutant (C51) that has a lesion in the 147-kDa subunit of RNA polymerase. These data support the contention that one or more late proteins are required for efficient resolution of concatemeric replicative intermediates. Identification of these concatemer resolution proteins may well be achieved through analysis of mutants that are defective in concatemer resolution but have little or no defect in late-protein synthesis. Two candidate genes have been identified. First, mutant C63 displays a concatemer phenotype that is identical to that of the severely late defective RNA polymerase mutants, yet it has normal kinetics of activation of late proteins and only slightly reduced amounts of some late proteins. The slight decrease in protein synthesis observed may possibly be accounted for by an indirect effect of unresolved concatemer products. A second mutant, 9383, with a phenotype similar to that of C63 was recently detected during screening of mutants obtained from S. Dales (10, 24). Preliminary mapping data placed the mutation in both C63 and 9383 within a late gene cluster in the *Hin*dIII-D/A junction region (46). The mutation in mutant C63 was localized to the left-most 1.5 kb of the *Hin*dIII-A region, consisting of three complete open reading frames (ORFs) corresponding to late proteins with predicted sizes of 17, 26, and 9 kDa, and part of the ORF encoding the major late core polypeptide P4b. The mutation in mutant 9383 was localized to a 1.9-kb *Bam*HI-*Xho*I region in *Hin*dIII-D which contains part of two ORFs encoding 63-kDa late polypeptides and the complete ORF encoding a 32-kDa protein which is expressed both early and late (25). A detailed analysis of these two mutants is in progress.

The weak defective late phenotype of two temperature-sensitive mutants was not accompanied by the presence of concatemers. The reduced quantity of DNA observed with one of these mutants (C50) may reflect defective viral DNA replication, which in turn could lead to a partial inhibition of late-protein synthesis following an initially wild-type kinetics and rate of late-protein synthesis. The defective gene product in C50 is nucleoside triphosphate phosphohydrolase I, a DNA-dependent ATPase (3, 38, 41), which has been postulated to function in transcription, DNA replication, and recombination. Further studies are needed to establish any such role(s).

Finally, the PFGE approach has proven very useful as a rapid and sensitive method to identify various types of replicative-intermediate DNA molecules and in its use of screening collections of temperature-sensitive mutants for specific defects in the conversion of these intermediates to a mature viral genome. Both PFGE and its sister method field inversion gel electrophoresis have the potential to become instrumental in identification of defective genomes, mapping (2), and detailed analysis of the interconversion of replicative-intermediate and mature genome structures of wild-type and temperature-sensitive mutants.

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