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Resolution of Vaccinia Virus DNA Concatemer Junctions Requires Late-Gene Expression

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Vaccinia virus replicates in the cytoplasm of infected cells, generating transient replicative intermediates containing the DNA for the terminal sequences as concatemeric junctions. The processing of the terminal sequences for a series of vaccinia virus conditional lethal mutants at the nonpermissive temperature was analyzed by restriction enzyme digestion and Southern blot hybridization of DNA isolated from infected cells. Three phenotypes were observed: DNA replication negative (Rep^-), DNA replication positive but concatemer resolution negative (Rep^+ Res^-), and DNA replication positive and concatemer resolution positive (Rep^+ Res^+). Interestingly, all six Rep^+ Res^- mutants from separate complementation groups were defective in late protein synthesis. Isatin beta-thiosemicarbazone, a drug that blocks late protein synthesis, also prevented resolution of concatemers. Orthogonal field gel electrophoresis of the DNA generated by the late defective mutants revealed a distribution of linear genome multimers. The multimers were processed into mature monomers after a shift to the permissive temperature in the presence of cytosine arabinoside for all the Rep^+ Res^- mutants except *ts*22, an irreversible mutant which cleaves RNA late in infection (R. F. Pacha and R. C. Condit, J. Virol. 56:395–403, 1985). Genome formation can be divided into two stages: DNA replication, which generates concatemers, and resolution, which processes concatemers into monomers with hairpin termini. Early viral genes are required for the former, and late viral genes are required for the latter.

Poxviruses are large double-stranded DNA viruses which replicate in the cytoplasm of infected cells. Vaccinia virus has a 185,000-base-pair genome with covalently continuous hairpin termini (3). It seems likely that the virus codes for all, or nearly all, of the factors required for its replication (21). Although little is known about the details of poxvirus DNA replication, the terminal sequences were detected as palindromes by restriction enzyme analysis of intracellular forms of rabbitpox virus (25) and vaccinia virus (4, 24). The association of these concatemeric molecules with the sites of DNA replication and their transient presence suggested that they were replicative intermediates. As a first step in the study of concatemer resolution, the junction fragment was cloned into bacterial plasmids and was shown to be a precise duplex copy of the terminal hairpin structure (17). Furthermore, the plasmids containing the junction were replicated in vaccinia virus-infected cells and converted into linear minichromosomes with vector DNA in the center and viral DNA hairpins at both ends (9, 18), providing direct evidence that monomer hairpin termini can be generated by the resolution of concatemer junctions.

To define the viral gene products required for the resolution of concatemers, we have studied the processing of the terminal genome sequences of a set of conditional lethal vaccinia virus mutants at the nonpermissive temperature. The mutants were grouped into three classes: replication negative (Rep^-), replication positive but resolution negative (Rep^+ Res^-), and replication positive and resolution positive (Rep^+ Res^+). The Rep^+ Res^- mutants were all defective in late protein synthesis (late defective) leading us to divide genome formation into two stages. In the first, early-gene products participate in viral DNA synthesis, generating replicative intermediates which contain concatemer junctions. In the second, a late-gene product(s) resolves the concatemers into monomers with hairpin termini.

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MATERIALS AND METHODS

Cell lines and viruses. BSC-1 cells, a continuous line of African green monkey cells, were grown in monolayer cultures in Eagle minimal essential medium (MEM; Quality Biologicals) supplemented with 10% fetal calf serum (GIBCO Laboratories). A set of well-characterized vaccinia virus (strain WR) temperature-sensitive mutants were provided by R. Condit (6, 7, 39). Virus stocks of temperature-sensitive (*ts*) mutants were prepared by low-multiplicity infections (less than 0.01 PFU per cell) of confluent BSC-1 monolayers at 31°C as described by Condit and Motyczka (6). The titers of the crude stocks were determined at both 31 and 40°C to ensure that each viral stock was conditionally defective at 40°C.

Analysis of viral DNA. Confluent 12- or 6-well monolayers of BSC-1 cells were infected with 0.5 or 1 ml of sonicated crude stock virus suspension at the multiplicity indicated below for each experiment. After incubation at room temperature for 2 h, the innoculum was replaced with 2 or 5 ml of MEM with 10% fetal calf serum. After incubation at 31 or 40°C for the specified time, the cell monolayers were scraped, pelleted by low-speed centrifugation, rinsed with phosphate-buffered saline, suspended in 50 μl of 0.15 M NaCl–0.02 M Tris hydrochloride (pH 8.0)–0.01 M EDTA and added to 250 μl of 0.02 M Tris hydrochloride (pH 8.0)–0.01 M EDTA–0.75% sodium dodecyl sulfate–0.4 to 0.8 mg of proteinase K (Boehringer Mannheim) per ml. After a 6-h incubation at 37°C, the samples were extracted with phenol, phenol:chloroform (1:1), and chloroform before precipitation with ethanol. The samples were suspended in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA (TE), passed through a 25-gauge needle to reduce viscosity, and digested with *Bst*EII (New England BioLabs, Inc.) at 37°C. The digests were electrophoresed through SeaKem ME (FMC Corp., Marine Colloids Div.) agarose by using E buffer (16) for neutral gels and 0.03 M NaOH–0.001 M EDTA for alkaline gels. Southern transfers and hybridizations were

TABLE 1. Description of temperature-sensitive (*ts*) mutants[a]

Complementation group	*ts* mutant	Phenotype	Complementation group	*ts* mutant	Phenotype
1	19	Normal	17	7	Late defective
2	21	Late defective	18	8	Normal
3	37	Normal	19	11	Normal
4	40	Normal	20	13	Normal
5	42	DNA replication negative	21	17	DNA replication negative
6	45	Normal	22	50	Normal
7	46	Normal	23	51	Normal
8	49	Normal	24	25	DNA replication negative
9	52	Normal	25	22	Late abortive
10	53	Late defective	26	33	Normal
11	56	Late defective	27	16	Normal
12	57	Normal	28	1	Normal
13	60	Normal	29	30	Normal
14	63	Late defective	30	18	Normal
15	35	Normal	31	12	Normal
16	6	Normal	32	27	Normal

[a] Complementation groups and phenotypes of mutants are derived from Condit et al. (7).

performed by using GeneScreen Plus (Du Pont Co.) as described by the manufacturer. Labeled probes were prepared by using a nick translation kit (Bethesda Research Laboratories), and fluorographs were made with Kodak XAR film.

Inhibitor drugs. Cytosine arabinoside (Sigma Chemical Co.) was dissolved at 4 mg/ml and added directly to MEM to a final concentration of 40 μg/ml. Rifampin (Mann Research Laboratories) was used at a final concentration of 100 μg/ml (1). Isatin beta-thiosemicarbazone (IBT; gift from R. Condit) was dissolved in acetone to 5 mg/ml, diluted with 4 volumes of 0.25 M NaOH immediately before use, and diluted 290-fold in MEM to bring the final concentration to 15 μM (27).

Orthogonal field gel electrophoresis. BSC-1 monolayers were infected at the indicated multiplicity, and after incubation at the times and temperatures described below for each experiment, the cells were scraped, pelleted by low-speed centrifugation, rinsed in TSE (100 mM NaCl, 50 mM EDTA, 20 mM Tris hydrochloride [pH 8.0]), and resuspended in TSE. The suspensions were mixed with a 0.5 volume of 2.0% LMP agarose (Bethesda Research Laboratories) maintained at 42°C, poured into molds (2 by 6 by 10 mm), and allowed to set on ice. The blocks contained approximately 2×10^6 or 2×10^7 cells per ml as indicated. The blocks were expelled into 1% *N*-lauroylsarcosine (sarcosinate)–0.5 M EDTA with 2 mg of proteinase K per ml and incubated at 37°C for 48 h with one buffer change after 24 h (40).

The orthogonal gel apparatus was constructed by T. Wellems and is similar to the one described by Carle and Olson (5). Slices from the agarose blocks were sealed in 4-mm thick 1% GTG agarose (FMC Corp.) gels and electrophoresed in 0.75× TBE (67 mM Tris base, 67 mM boric acid, 1.5 mM EDTA). Buffer was recirculated and maintained at 15°C. Pulsing times and voltages are given in the figure legends. The gels were stained with ethidium bromide and, after photography, were exposed to UV light for approximately 5 min before the DNA was transferred to GeneScreen Plus.

Analysis of concatemers. An orthogonal field agarose gel of BSC-1 cells obtained 24 h postinfection with *ts*21 was stained with ethidium bromide, and the agarose blocks containing the bands were excised. Ethidium bromide was extracted with four washes of 2-butanol saturated in 1 M NaCl–10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA. After four washes with TE, the blocks were incubated in 0.5 ml of *Bst*EII restriction enzyme digestion buffer overnight. The blocks were placed in 0.2 ml of fresh *Bst*EII restriction enzyme digestion buffer and digested with 40 U of *Bst*EII for 14 h at 37°C. The digestion products were electrophoretically transferred onto DEAE paper (DEAE 81; Whatman, Inc.), eluted with 1.25 M NaCl–7.5 mM Tris hydrochloride (pH 8.0)–0.75 mM EDTA, and precipitated with ethanol using tRNA as carrier nucleic acid. The samples were resuspended in TE, electrophoresed through agarose, transferred to GeneScreen Plus, and hybridized with terminal sequences labeled with ^{32}P by nick translation. Fluorographs of the filter were made with Kodak XAR film, and optical scanning was performed with an LKB Ultrascan XL densitometer.

Analysis of concatemer junctions. BSC-1 cells were infected with *ts*21 or wild-type virus, harvested at 2×10^6 cells per agarose block, and lysed as described above. The blocks were washed four times with TE and incubated with 0.5 ml of *Hin*dIII restriction enzyme digestion buffer (New England BioLabs) for 16 h at room temperature. The buffer was replaced with 0.2 ml of fresh *Hin*dIII restriction enzyme digestion buffer, and the DNA was digested with 60 U of *Hin*dIII for 8 h at 37°C. The blocks were sealed in a 1% SeaKem GTG agarose gel and subjected to orthogonal field gel electrophoresis. The gel was stained with ethidium bromide and, after photography, was exposed to UV light for 5 min before the DNA was transferred to a nylon membrane. Lambda markers were prepared as described by Wellems et al. (40).

RESULTS

Concatemer junction processing by temperature-sensitive mutants. To identify the viral gene products that participate in the processing of concatemeric forms of vaccinia virus DNA, we studied the conversion of the concatemer junction to mature hairpin termini in cells infected with temperature-sensitive mutants previously described by Condit and coworkers (6, 7, 39). The representative mutant from each complementation group used in this study is described in Table 1.

The time course of formation and resolution of concatemer junctions at 40°C, the nonpermissive temperature for the temperature-sensitive mutants, was first determined for wild-type virus as a base line for further studies. DNA, isolated at specified times after infection, was digested with

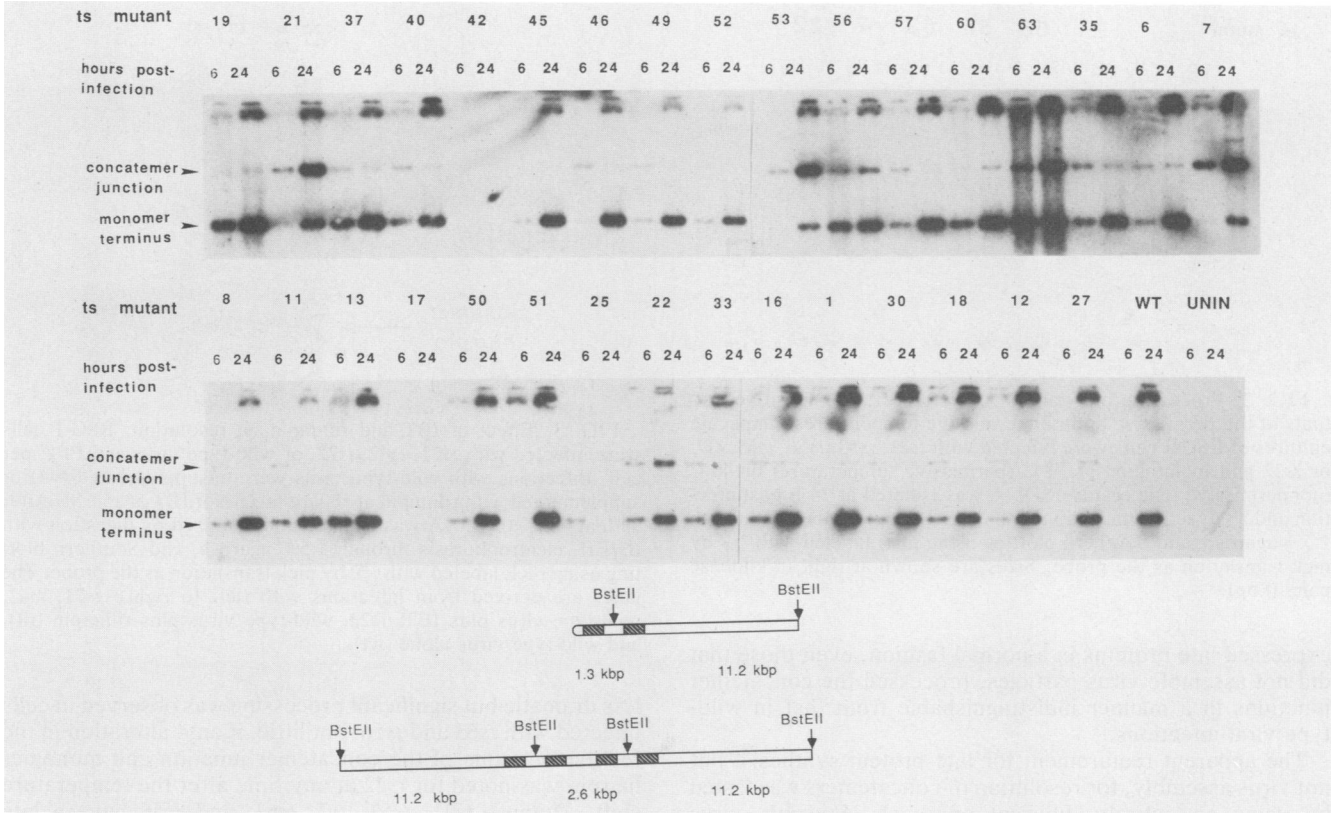

FIG. 1. Processing of viral DNA by temperature-sensitive mutants at 40°C. BSC-1 cells were mock infected (UNIN), infected with wild-type virus (WT), or with one of the temperature-sensitive mutants at 5 PFU per cell and maintained at 40°C. DNA was isolated 6 or 24 h postinfection and analyzed by digestion with *Bst*EII, electrophoresis through 1.5% agarose, and Southern blotting using pRE labeled with ^{32}P by nick translation as the probe. The plasmid pRE contains part of the hairpin loop and one copy of the direct repeats. Below is a representation of the mature terminus and concatemer junction of vaccinia virus, with the direct repeats shown as darkened regions. kbp, Kilobase pairs.

restriction endonuclease *Bst*EII, electrophoresed through agarose, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled probe containing terminal genomic sequences. As shown in the bottom panel of Fig. 1, DNA segments derived from concatemer junctions are twice the length of fragments from mature genomes, e.g., 2.6 versus 1.3 kilobase pairs (kb), after *Bst*EII digestion. A larger 11.2-kb fragment, that also hybridized to the probe, is derived from the proximal internal sequences and serves as a useful standard. Maximal amounts of concatemer junction were observed approximately 6 h postinfection; by 24 h the terminal sequences were almost exclusively present as mature hairpin termini.

Each of the temperature-sensitive mutants described in Table 1 was used to infect BSC-1 cells at a multiplicity of 5 PFU per cell, and the cells were incubated at the conditional lethal temperature of 40°C. On the basis of the results with wild-type virus, the DNA was isolated 6 and 24 h after infection and analyzed for terminal sequences as described above (Fig. 1). Three phenotypes were observed. Mutants *ts*42, *ts*17, and *ts*25, which were classified as DNA replication negative (7), were unable to replicate viral DNA at the conditional lethal temperature. The majority of the mutants replicated their DNA, and the 1.3-kb terminal fragment was present almost exclusively at 24 h postinfection. However, with mutants *ts*21, *ts*53, *ts*56, *ts*63, *ts*7, and *ts*22, both the unresolved 2.6-kb concatemer junction and the 1.3-kb hairpin terminus were present at 24 h postinfection.

The mutants defective in the ability to process the concatemer junction at the nonpermissive temperature of 40°C were also tested at the permissive temperature of 31°C to be certain that the defect was associated with the temperature-sensitive mutation. Duplicate cultures of BSC-1 cells were infected with *ts*21, *ts*53, *ts*56, *ts*63, *ts*7, and *ts*22, incubated at 31 or 40°C, and harvested after 24 h. The DNA was isolated and analyzed for the presence of monomer termini and concatemer junctions (Fig. 2). For each mutant, accumulation of the concatemer junction was most pronounced at the conditional defective temperature. Conversion of the junction fragment to a monomer hairpin proceeded normally at 31°C, although with mutant *ts*63, some concatemer junction remained at 24 h.

Resolution required late-gene product(s). The temperature-sensitive mutants have been previously grouped into several major phenotypes (6, 7). Mutants have been classified as (i) normal when they synthesize DNA and protein in a manner indistinguishable from that of wild-type virus, (ii) DNA replication negative, and (iii) defective or abortive in late protein synthesis (late defective or late abortive). As expected, the DNA replication-negative mutants *ts*42, *ts*17, and *ts*25 made little or no DNA after infection at 40°C (Fig. 1). The phenotypes of the mutants which replicate DNA and accumulate concatemer junction at the conditional defective temperature are shown in Table 1. In each case the accumulation of the concatemer junction is associated with defects in late protein synthesis. By contrast, all mutants which

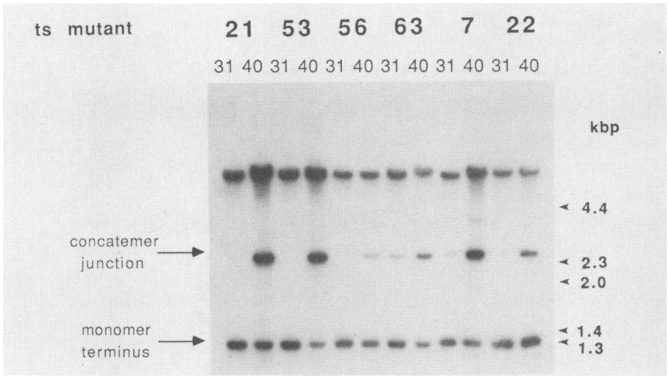

FIG. 2. Processing of viral DNA by temperature-sensitive mutants at the permissive and nonpermissive temperatures. Duplicate cultures of BSC-1 cells were infected with *ts*21, *ts*53, *ts*56, *ts*63, *ts*7, or *ts*22 and maintained at 31°C (permissive temperature) or 40°C (nonpermissive temperature). DNA was isolated at 24 h postinfection and analyzed by digestion with *Bst*EII, electrophoresis through 1.5% agarose, and Southern blotting using pRE labeled with ^{32}P by nick translation as the probe. Sizes are shown at right in kilobase pairs (kbp).

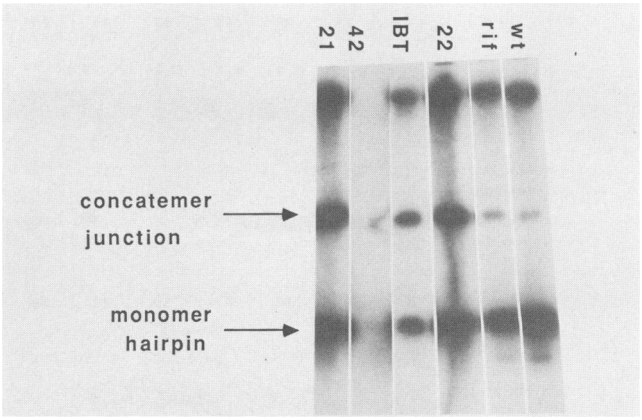

FIG. 3. Effect of IBT and rifampin on resolution. BSC-1 cells were infected with *ts*21, *ts*42, *ts*22, or wild-type virus at 5 PFU per cell. Infections with wild-type virus were unsupplemented (wt) or supplemented with rifampin at 100 μg/ml (rif) or IBT at 15 μM. After 24 h at 40°C, the DNA was isolated and analyzed by digestion with *Bst*EII, electrophoresis through 1.5% agarose, and Southern blotting using pRE labeled with ^{32}P by nick translation as the probe. The lanes are derived from infections with (left to right): *ts*21, *ts*42, wild-type virus plus IBT, *ts*22, wild-type virus plus rifampin (rif), and wild-type virus alone (wt).

expressed late proteins in a normal fashion, even those that did not assemble virus particles, processed the concatemer junctions in a manner indistinguishable from that in wild-type viral infections.

The apparent requirement for late protein synthesis, but not virus assembly, for resolution of concatemers was tested by using an entirely different approach. Vaccinia virus infection is blocked at different stages of the infectious cycle by inhibitor drugs. IBT, which inhibits poxvirus replication (27, 35), severely restricts the synthesis of late viral proteins (28, 43). The antiviral agent rifampin does not interfere with macromolecular synthesis (22) but is associated with the blockage of envelope formation and morphogenesis (23, 26, 29). BSC-1 cells were infected with wild-type vaccinia virus and treated with rifampin or IBT, and the processing of the concatemer junction at 40°C was compared with that in infections with wild type, *ts*42, *ts*21, and *ts*22 (Fig. 3). Resolution of the concatemer junction occurred equally well with wild-type virus in the presence or absence of rifampin. Treatment with IBT, however, led to the accumulation of the concatemer junction fragment similar to that observed for *ts*21 and *ts*22 at 40°C. It has previously been demonstrated that the effects of IBT on vaccinia virus-infected cells closely resemble those observed for *ts*22 (27). The results with the virus inhibitors are consistent with the requirement for late viral gene product(s) for concatemer resolution.

Resolution did not require concurrent DNA replication. If the concatemer junctions observed at the conditional lethal temperature for the late defective mutants were replicative intermediates, one would predict that these structures would be converted to mature monomer hairpins once the infection was shifted to the permissive temperature. BSC-1 cells were infected with the mutants *ts*21, *ts*53, *ts*56, *ts*63, *ts*7, and *ts*22 at a multiplicity of 1 PFU per cell and incubated at 40°C for 14 h. The cultures were shifted to 31°C, cells were harvested at specified times, and the DNA was isolated and analyzed for monomer ends and concatemer junctions. The results for *ts*21 and *ts*53 are shown in Fig. 4. The accumulated concatemer junctions decreased and the monomer ends increased after the temperature was shifted to 31°C, so that by 26 h later most of the terminal sequences were detected as monomer hairpins. Similar results were observed for *ts*7; less dramatic but significant processing was observed in cells infected with *ts*63 and *ts*56, but little, if any, alteration in the relative amounts of the concatemer junction and monomer hairpin was noted for *ts*22 at any time after the temperature shift. Mutants *ts*21, *ts*53, *ts*7, *ts*63, and *ts*56 have a late defective phenotype, whereas mutant *ts*22 has a late abortive phenotype. The latter has been shown to degrade RNA late in infection, irreversibly aborting viral infection (27). Thus, conversion of the concatemer junction to a monomer hairpin occurs under conditions in which the temperature shift to the permissive leads to the production of late viral gene products and is consistent with the processing of replicative intermediates.

To determine the contribution of de novo vaccinia virus DNA replication to the processing of the concatemer junc-

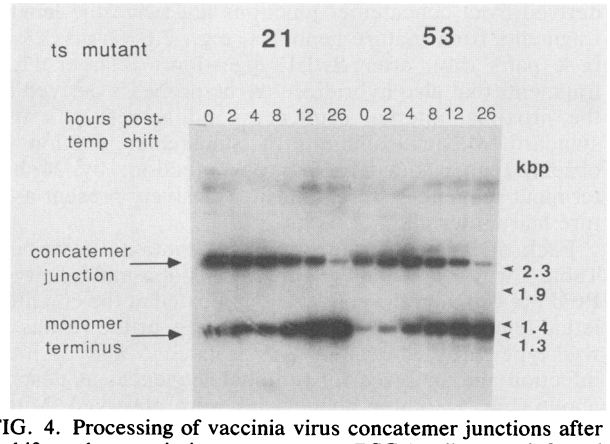

FIG. 4. Processing of vaccinia virus concatemer junctions after the shift to the permissive temperature. BSC-1 cells were infected with *ts*21 or *ts*53 at 1 PFU per cell, incubated at 40°C for 14 h, and then shifted to 31°C, the permissive temperature. Cells were harvested 0, 2, 4, 8, 12, and 26 h after the temperature shift, and the DNA was isolated and analyzed by *Bst*EII digestion, electrophoresis through 1.5% agarose, and Southern blotting using pRE labeled with ^{32}P by nick translation as the probe. Sizes are shown at right in kilobase pairs (kbp).

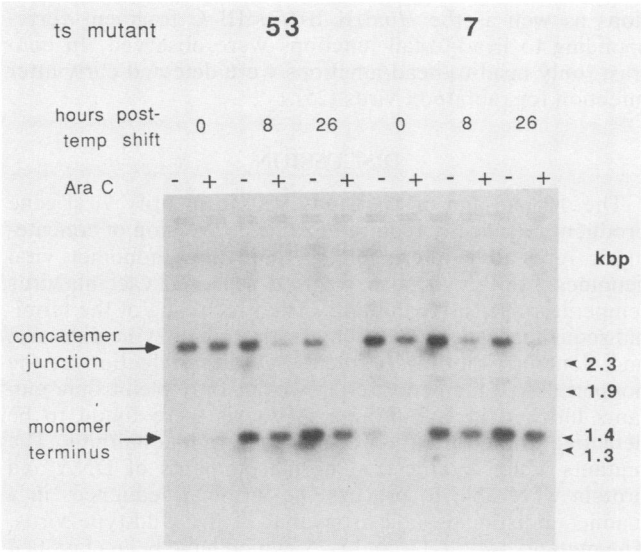

FIG. 5. Processing of vaccinia virus concatemer junctions in the presence of ara-C. BSC-1 cells were infected with *ts*53 or *ts*7 at 1 PFU per cell and incubated at 40°C for 14 h. Half of the cultures were supplemented with 40 μg of ara-C per ml, and after another 1.5 h, all samples were shifted to 31°C. Cells were harvested 0, 8, and 26 h after the temperature shift. DNA was isolated and analyzed by *Bst*EII digestion, electrophoresis through 1.5% agarose, and Southern blotting using pRE labeled with ^{32}P by nick translation as the probe. Sizes are shown on the right in kilobase pairs (kbp).

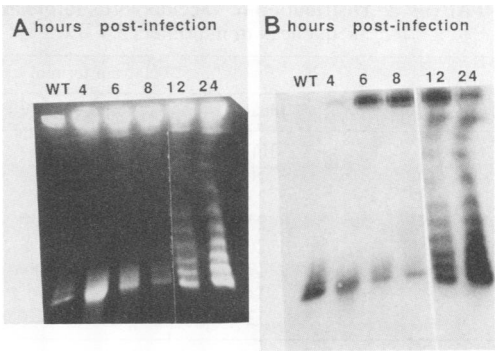

FIG. 6. Analysis by orthogonal field gel electrophoresis of viral DNA produced during *ts*21 infection. BSC-1 cells were infected with *ts*21 at 5 PFU per cell, cells were harvested at 4, 6, 8, 12, and 24 h postinfection, and agarose blocks containing DNA from 2×10^7 cells were prepared as described in Materials and Methods. Samples were electrophoresed on a 1% agarose gel at 80 V for 130 h with a switching time of 12 min. The gel was stained with ethidium bromide (A), and the DNA was transferred to a nylon membrane and hybridized with vaccinia virus DNA labeled with ^{32}P by nick translation (B). DNA isolated 24 h postinfection by using wild-type virus appears in lanes WT.

tion, cytosine arabinoside (ara-C), a potent inhibitor of vaccinia virus DNA polymerase, was included in temperature shift experiments. Duplicate cultures of BSC-1 cells were infected with the late protein synthesis-defective mutants *ts*21, *ts*53, and *ts*7 and incubated at 40°C for 14 h. Half of the cultures were treated with 40 μg of ara-C per ml and after 90 min were shifted to 31°C. Samples were removed 0, 8, and 26 h later, and the DNA was isolated and analyzed for monomer ends and concatemer junctions. The results for mutants *ts*53 and *ts*7 are shown in Fig. 5. Conversion of the concatemer junction to a monomer hairpin was observed for both mutants in the presence or absence of ara-C. The samples treated with ara-C contain even less residual concatemer junction than the analogous untreated samples not inhibited for DNA replication. The data for *ts*21 were equivalent to those for *ts*53 and *ts*7. We concluded that the conversion of concatemer junctions to monomer hairpins does not require concurrent DNA synthesis.

Accumulation of linear multimers in late defective infections. To directly analyze the products generated in cells infected with the late defective mutants, we utilized orthogonal field gel electrophoresis, a technique used to separate large DNA fragments on the basis of multiple reorientations in alternating perpendicular electric fields (33, 37). BSC-1 cells infected with the late defective mutant *ts*21 were lysed, and the DNA was deproteinized within agarose plugs prior to electrophoresis through an orthogonal field gel. After being stained with ethidium bromide (Fig. 6A), the DNA was transferred to GeneScreen Plus and hybridized with nick-translated vaccinia virus DNA (Fig. 6B). The viral DNA was composed of monomer genomes and molecules which did not enter the gel for up to 8 h postinfection. At 12 and 24 h postinfection, the mutant viral DNA was composed of a ladder of evenly spaced fragments. Orthogonal field agarose gels with shorter pulse times demonstrated that the lowest four viral DNA fragments were each separated by approximately 4.5 bacteriophage lambda multimers, which is equivalent to slightly more than a 200-kb spacing (data not shown). Equivalent arrays of multimeric viral DNA were observed late after infection of cells infected with the mutants *ts*53 and *ts*7. In contrast, DNA from wild-type vaccinia virus-infected cells was present almost exclusively as monomers at 24 h (Fig. 6).

To determine the nature of the resolved DNA molecules detected at 24 h after mutant-virus infection, agarose plugs containing the eight fastest-migrating fragments from a gel equivalent to that shown in Fig. 6 were excised and treated with *Bst*EII. The DNA digestion products were resolved by agarose gel electrophoresis and transferred to a nylon membrane. The blots were hybridized to a ^{32}P-labeled probe containing terminal sequences, and fluorographs were scanned by using a densitometer. The areas under the concatemer junction and monomer hairpin were determined, and the percentage of the total that was monomer is shown in Table 2. Since a linear multimer will always contain two hairpin ends and $N - 1$ concatemer junction fragments and the concatemer junction will hybridize to twice as much probe as the monomer hairpin, the percentage of the hybridized label in the terminal fragments for an N-mer is $100\{2/[2 + 2(N - 1)]\}$ or $100/N$. The percentage of the total probe found in the hairpin termini for each of the viral fragments is consistent with a set of linear integral multimers from monomer to octomer in genome length.

The processing of DNA intermediates after the shift to the permissive temperature was investigated by using orthogonal field gel electrophoresis. Agarose plugs were prepared from BSC-1 cells which had been infected with *ts*21 at 40°C for 14.5 h. The temperature was shifted to 31°C, and the cells were harvested at specified times. The samples were electrophoresed through an orthogonal field gel, blotted, and probed with vaccinia virus DNA. The DNA molecules trapped at the origin and array of linear multimers present 14.5 h after infection at 40°C were quantitatively converted into monomer vaccinia virus genomes within 24 h after the temperature shift (Fig. 7).

TABLE 2. Distribution of vaccinia virus terminal sequences in multimers

N-mer	% Hairpin termini	
	Ideal[a]	Observed[b]
1	100	84
2	50	53
3	33	41
4	25	22
5	20	17
6	17	12
7	14	15
8	12	13

[a] Percentage of labeled material hybridized to terminal fragment is 100/N as described in Results.

[b] Values were determined by densitometric analysis of Southern blots using a probe for terminal sequences as described in Materials and Methods.

Analysis of viral concatemer junctions. BSC-1 cells were infected with the late defective mutant *ts*21 at 5 or 30 PFU per cell and harvested after 14 or 24 h at 39.5°C. Also, BSC-1 cells were infected with wild-type virus at 30 PFU per cell and harvested after 24 h at 37°C. For each infection, the cells were lysed and the DNA was deproteinized within agarose blocks. The samples were digested with *Hin*dIII, electrophoresed through an orthogonal field agarose gel, and transferred to a nylon membrane. The filter was hybridized with pRE, a plasmid containing sequences present at both ends of the genome, and pHSB and pHSC, plasmids containing unique sequences from the terminal *Hin*dIII B and *Hin*dIII C fragments (19; Fig. 8). The filter was treated with 0.5 M NaOH and fluorographed between each hybridization to ensure that the probe had been removed. The lower portion of Fig. 8 shows the *Hin*dIII fragments containing the terminal sequences expected from head-to-head or head-to-tail concatemers. The set of hybridizations demonstrates that *ts*21 DNA contained both types of concatemer junctions 14 and 24 h after infection. The *Hin*dIII B-*Hin*dIII B and *Hin*dIII C-*Hin*dIII C fragments due to head-to-head junctions as well as the *Hin*dIII B-*Hin*dIII C fragment corresponding to head-to-tail junctions were observed. In contrast, only head-to-head junctions were detected early after infection for rabbitpox virus (25).

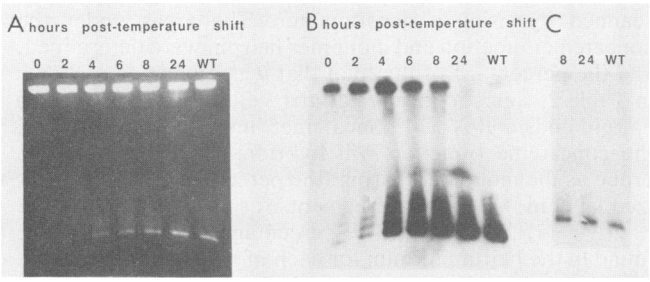

FIG. 7. Analysis by orthogonal gel electrophoresis of the processing of vaccinia virus DNA in the presence of ara-C. BSC-1 cells were infected with *ts*21 at 5 PFU per cell, incubated at 40°C for 14.5 h, and supplemented with 40 μg of ara-C per ml. After an additional 1.5 h, the cultures were shifted to 31°C, samples were harvested at 0, 2, 4, 6, 8, and 24 h after the temperature shift, and agarose blocks containing DNA from 2×10^6 cells were prepared as described in Materials and Methods. The samples were electrophoresed through a 1% agarose gel at 80 V for 92 h by using a switching time of 12 min. The gel was stained with ethidium bromide (A), and the DNA was transferred to a nylon membrane and hybridized with vaccinia virus DNA labeled with ^{32}P by nick translation (B). Panel C is a shorter exposure of the rightmost three lanes. The sample on the far right of the gel contains DNA isolated 24 h after infection with wild-type virus (WT).

DISCUSSION

The original aim of this study was to identify viral gene products specifically required for the conversion of concatemeric replicative intermediates to mature monomer viral genomes. For this purpose, we used a set of 32 vaccinia virus temperature-sensitive mutants. The processing of the terminal sequences was analyzed by restriction enzyme digestion and Southern blotting of the DNA after infection at the nonpermissive temperature. However, only pleiotropic mutants blocked in late protein synthesis were found to be defective in the formation of mature terminal hairpins. The mutants which synthesized normal amounts of DNA and protein were able to process the terminal sequences in a manner indistinguishable from that of the wild-type virus. The mutants *ts*42, *ts*17, and *ts*25, which have been classified as DNA replication negative (7), did not synthesize replicative intermediates. The mutants *ts*21, *ts*53, *ts*56, *ts*63, and *ts*7, which have late defective phenotypes (7), and *ts*22, which has a late abortive phenotype (7, 27), were shown to accumulate the concatemer junction after infection at the conditional lethal temperature. The late defective and abortive phenotypes have been associated with diminution of all late proteins. The late defective mutants *ts*21, *ts*53, and *ts*7 have been mapped to three RNA polymerase subunits (13a, 34, 39a), providing a rationale for the pleiotropic effect on late protein synthesis. The late abortive mutant *ts*22 extensively cleaves RNA late after infection, effectively abrogating translation (27). It was not surprising that the mutants accumulated different amounts of concatemer junction, since the late defective phenotype is quite heterogenous. The most stringent examples of the phenotype, *ts*53 and *ts*7 (7), most poorly resolved the concatemer junction.

The inability to detect mutants which solely inhibit the processing of concatemer junctions could arise from several scenarios. Although most unlikely, the resolution of the concatemer junctions could be nonessential, so that thermolabile mutants would not be isolated. Since the mutant library only contains 32 complementation groups, it may not include those with lesions in the gene products specifically required for resolution. The method of generation and selection of mutants may preclude the isolation of mutants in certain genes. Another possibility is that the genes used for resolution may be utilized for other steps in virus metabolism, such as late transcription or translation resulting in pleiotropic phenotypes.

The processing of concatemeric intermediates in herpesvirus is apparently linked to the packaging of DNA into empty capsids (14, 32, 36). The requirement for late vaccinia virus protein synthesis for concatemer processing could reflect a need for preformed viral procapsids. However, treatment with rifampin, which abrogates particle formation, does not interfere with concatemer resolution. Furthermore, some of the mutants which synthesize normal amounts of DNA and proteins were not observed to form enveloped structures by electron microscopy (R. C. Condit, personal communication), although they readily processed concatemeric replicative intermediates.

The processing of bacteriophage T7 DNA concatemers has been shown to require endonuclease activity and DNA polymerase (41). To determine the contribution of vaccinia

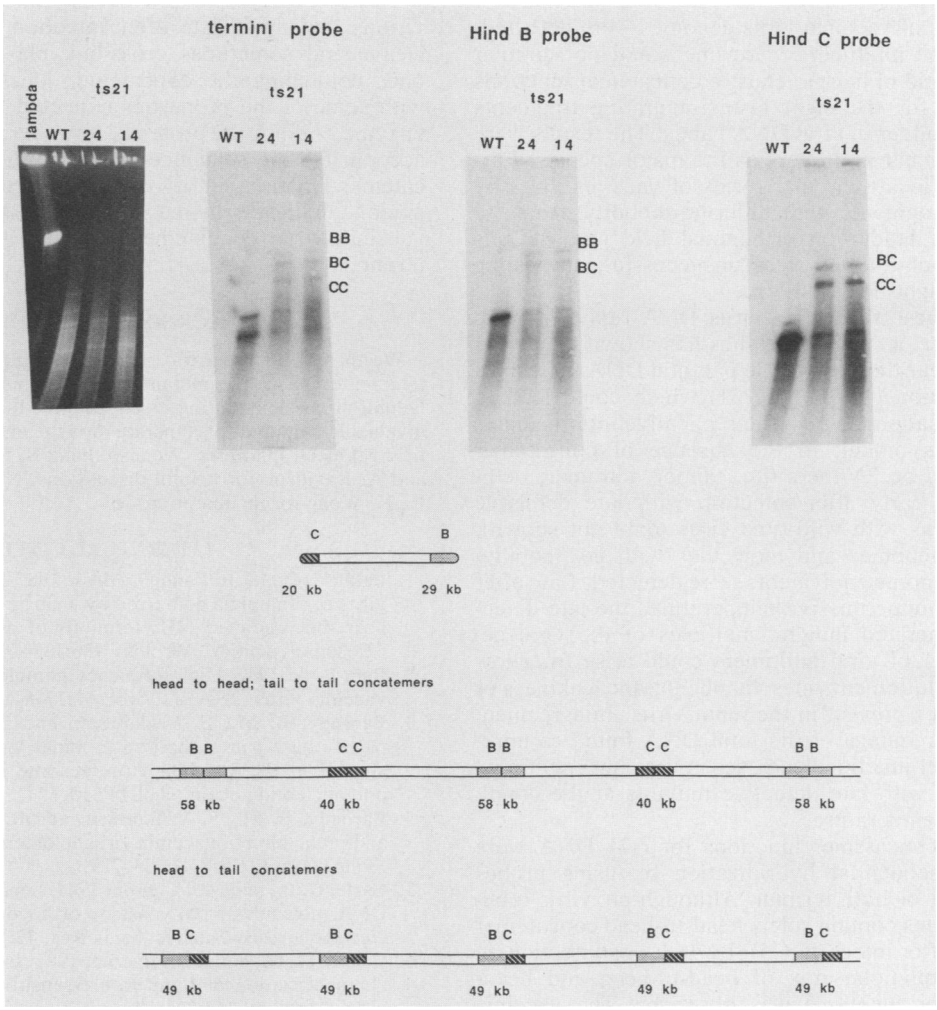

FIG. 8. Analysis by orthogonal gel electrophoresis of the concatemer junctions in viral DNA produced during *ts*21 infection. BSC-1 cells were infected with wild-type virus and harvested 24 h after infection (WT) or with *ts*21 and harvested 14 or 24 h after infection. Agarose blocks containing 2×10^6 cells were lysed, and the DNA was digested as described in Materials and Methods. The samples were electrophoresed through 1% GTG agarose at 200 V for 15 h with a switching time of 2 s. The gel was stained with ethidium bromide (left panel), and the DNA was transferred to a nylon membrane. The filter was hybridized with pRE, a plasmid containing sequences present at both ends of the genome (termini probe); pHSB, a plasmid derived from the vaccinia virus *Hin*dIII B fragment (Hind B probe); and pHSC, a plasmid derived from the *Hin*dIII C fragment (Hind C probe). The probe was removed by treatment with 0.5 NaOH between each hybridization. The junction fragments arising from head-to-head (BB and CC) and head-to-tail (BC) concatemers are indicated on the right of each fluorograph. The far left lane in the leftmost panel contains lambda DNA. Below the fluorographs is a representation of the expected *Hin*dIII digestion fragments containing the terminal sequences found for head-to-head and head-to-tail concatemers. The top figure shows the vaccinia virus genome with the *Hin*dIII B and *Hin*dIII C fragments shaded. The bottom two figures denote the *Hin*dIII sites bordering the terminal sequences in head-to-head or head-to-tail concatemers.

virus DNA polymerase to resolution, ara-C, a potent inhibitor of the viral DNA polymerase, was added to cells after infection with temperature-sensitive mutants at the nonpermissive temperature and the terminal sequences were determined after the sample was shifted to the permissive temperature. Efficient processing of the concatemer junction to a monomer hairpin and generation of infectious temperature-sensitive virus were observed in the presence of ara-C. The samples were also digested with *Bst*EII, electrophoresed through an alkaline agarose gel, transferred to a nylon membrane, and hybridized with nick-translated terminal DNA sequences. A predominant species at approximately 2,600 nucleotides, corresponding to full-length concatemer junction or covalently continuous terminal hairpin, but no shorter fragments diagnostic of nicked intermediates, was detected for each sample at every time point. Thus, the conversion of concatemer junctions to monomer hairpins requires little, if any, concurrent viral DNA polymerase activity.

The requirement for late protein synthesis was also demonstrated by using bacterial plasmids containing the concatemer junction. Such plasmids are converted into linear minichromosomes with sealed hairpin termini after transfection into cells infected with vaccinia virus (9, 18). However, plasmids containing the junction fragment are poorly resolved into linear minichromosomes when transfected into cells infected with the late defective or abortive temperature-sensitive mutants at the nonpermissive temperature (19).

The structures of the DNA species that accumulate in cells infected with the late defective mutants at the conditional lethal temperature were investigated by using orthogonal field gel electrophoresis. A series of bands corresponding in

size to multimers of vaccinia virus genomes were detected. To prove that the multimers were linear and not circular molecules, the ratio of hairpin ends to concatemer junctions was determined for the eight fastest-migrating fragments constituting the ladder of viral DNA bands. The results were consistent with linear multimers as the major species. Further attempts to locate circular forms of vaccinia virus by detecting viral fragments with differing mobility against a lambda multimer ladder on orthogonal field agarose gels using different pulse times were unsuccessful, suggesting that the forms might not exist.

The initial stages of vaccinia virus DNA replication are unclear. However, a series of studies has shown that nicks are introduced near the ends of the parental DNA molecules early after infection (10, 11, 30, 31) which could lead to self-primed replication (3, 25). The replicative intermediates generated in this model, in the absence of concatemer resolution, would be 2*N*-mers (i.e., dimer, tetramer, octomer). However, early after infection with late defective mutants as well as with wild-type virus (data not shown), only monomer genomes and molecules with gel mobility slower than 12 genome equivalents were detected. Late after infection at the nonpermissive temperature, the late defective mutants generated integral multiples of the viral genome. The ladder of viral multimers could arise from low levels of the resolution enzymes via phenotypic leakiness or packaged enzymes present in the input virus. In agreement with this, the percentage of the total DNA found as unresolved concatemer junction decreased at very high multiplicities of infection with late defective mutants at the conditional defective temperature.

The types of concatemer junctions for *ts*21 DNA were determined by sequential hybridization by using probes specific for either or both termini. Although poxvirus replicative intermediates contain solely head-to-head concatemer junctions early after infection (25), by 14 h postinfection an approximately equimolar mix of head-to-head and head-to-tail concatemer junctions was observed. The vaccinia virus genome contains inverted terminal repeats of 10 kb (42). The mix of junction fragments could arise from homologous recombination between the terminal sequences embedded within the arrays of viral multimers. The equimolar distribution of the two conformers implies that the level of recombination is high enough to generate randomly oriented genomes within the multimer array.

Also, the ladder of viral genomes could arise via homologous recombination between replicative intermediates, generating a random set of *N*-mers. Recombination within the terminal sequences between two molecules of length x and y will yield products of $x + n$ and $y - n$. High levels of recombination have been demonstrated in cells infected with wild-type (2, 12, 38) or late defective (M. Merchlinsky, submitted for publication) vaccinia virus. The DNA species observed early after infection could be multimers of greater than 12 genome equivalents made too quickly to be detected or heterogenous branched replicative intermediates generated by a mechanism such as strand invasion (13, 20). Studies are presently under way to discriminate between such models.

In conclusion, the results described in this report allow us to divide genome formation into two stages: DNA replication, which generates concatemeric replicative intermediates, and resolution, which converts concatemers into monomers with hairpin termini. Expression of early genes is required for the former, and expression of late viral genes is required for the latter. Recently, an enzyme purified from virions and made late after infection has been shown to cleave and sometimes cross-link plasmids (15), including ones containing the concatemer junction (17), consistent with some of the properties expected for a nicking-closing enzyme. Additional proteins, however, might be needed to account for the sequence specificity for resolution of concatemer junctions in vivo (8, 19). Further efforts are being made to delineate the mechanism used for the conversion of vaccinia virus concatemer junctions to monomer hairpin termini.

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