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Genetics

Vaccinia virus DNA replication: Two hundred base pairs of telomeric sequence confer optimal replication efficiency on minichromosome templates

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ABSTRACT **Vaccinia virus is a complex DNA virus that exhibits significant genetic and physical autonomy from the host cell. Most if not all of the functions involved in replication and transcription of the 192-kb genome are virally encoded. Although significant progress has been made in identifying trans-acting factors involved in DNA synthesis, the mechanism of genome replication has remained poorly understood. The genome is a linear duplex with covalently closed hairpin termini, and it has been presumed that sequences and/or structures within these termini are important for the initiation of genome replication. In this report we describe the construction of minichromosomes containing a central plasmid insert flanked by hairpin termini derived from the viral genome and their use as replication templates. When replication of these minichromosomes was compared with a control substrate containing synthetic hairpin termini, specificity for viral telomeres was apparent. Inclusion of ≥200 bp from the viral telomere was sufficient to confer optimal replication efficiency, whereas 65-bp telomeres were not effective. Chimeric 200-bp telomeres containing the 65-bp terminal element and 135 bp of ectopic sequence also failed to confer efficient replication, providing additional evidence that telomere function is sequence-specific. Replication of these exogenous templates was dependent upon the viral replication machinery, was temporally coincident with viral replication, and generated covalently closed minichromosome products. These data provide compelling evidence for specificity in template recognition and utilization in vaccinia virus-infected cells.**

Poxviruses are complex DNA viruses that replicate in the cytoplasm of infected cells. This unusual physical compartmentalization is coupled with significant genetic autonomy from the host; the 192-kb viral genome encodes most if not all of the functions required for DNA replication and three temporally regulated classes of gene expression (1–4). With regard to DNA replication, there has been steady progress in applying both genetic and biochemical approaches to unravel the repertoire of proteins involved. The DNA polymerase and processivity factor, DNA-independent NTPase, serine/threonine protein kinase, thymidine kinase, thymidylate kinase, ribonucleotide reductase, uracil DNA glycosylase, dUTPase, topoisomerase I, and DNA ligase have demonstrated or presumed roles in DNA synthesis (5, 6).

A working model for DNA replication has emerged that incorporates the few experimental details available regarding the mechanism of genome replication (4, 6, 7). The DNA genome is a linear duplex with covalently closed hairpin termini, and replicative synthesis is presumed to begin from a nick introduced near a genomic terminus. This nick exposes a 3′OH group which serves as a primer for strand-displacement synthesis. The end product of this synthesis is a tail/tail dimer that is then resolved to monomeric progeny genomes. Although variants of this model have existed for many years, there has been virtually no progress to date in identifying the site of replication initiation or in defining what sequences and/or conformation define a poxviral replication template.

This lack of progress has in part been due to an apparent conflict in the literature. Identification of the site of replication initiation was addressed by synchronizing infected cultures just prior to DNA replication with the use of temperature-sensitive mutants (indicated by *ts*), and then permitting replication to begin in the presence of [^{3}H]thymidine (8, 9). Analysis of the gradient of thymidine incorporation generated indicated that initiation occurred within 200 bp of the tip of the hairpin terminus (9). More recently, however, any supercoiled template was found to replicate to some extent in poxvirus-infected cells, and this replication was not enhanced by the inclusion of viral sequences on the plasmid template (10–12). These data suggested that there might not be any specificity in template recognition.

The studies described in this report were designed to move beyond this impasse. With the presumption that both DNA sequence and structure would contribute to template recognition, we have designed minichromosome templates that retain the topology of the natural viral chromosome. With this approach we have been able to define and delineate genomic elements that are necessary and sufficient to comprise a good replication template.

MATERIALS AND METHODS

Cells and Virus. Mouse L cells, African green monkey BSC40 cells, and wild-type vaccinia virus (WR strain) were maintained and propagated as described (13). For propagation of *ts*42 and *ts*17 (14–16), originally provided by R. Condit (University of Florida), 32°C and 39.5°C served as the permissive and nonpermissive temperatures, respectively.

Materials. Restriction endonucleases and exonuclease III were purchased from Boehinger Mannheim; concentrated T4 DNA ligase (2,000,000 units/ml) was obtained from New England Biolabs. Lipofectin was acquired from GIBCO/BRL Life Technologies (Gaithersburg, MD) and used according to the manufacturer's instructions.

Plasmids. pBSIIKS^{-Sal} was created by eliminating the *Sal*I restriction site of the pBSIIKS (Stratagene). pBSIIKS was digested with *Sal*I and the termini were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of dNTPs; the blunt-ended pBSIIKS linear DNA was then religated. pSD and pSV9 were kindly provided by Michael Merchlinsky (U.S. Department of Agriculture) (17). pSD is a puc13 derivative containing a 7.4-kb insert derived from the vaccinia virus concatameric junction. pSV9 is a dimeric,

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Abbreviation: hpi, hours postinfection.

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symmetrical plasmid containing two copies of a 2.6-kb insert derived from the vaccinia virus concatameric junction and two copies of puc13 DNA.

The pHS plasmid contains a 414-bp insert derived from the vaccinia virus telomeric hairpin. A 414-bp viral concatameric junction fragment was excised from pSV9 with *Hinf*I, and the termini were filled-in with the Klenow polymerase. The blunt-ended fragment was subsequently ligated to *Sal*I linkers, and the *Sal*I-digested fragment was cloned into pBSIIKS DNA at the *Sal*I site.

The p150 plasmid contains a 300-bp palindromic insert generated by two PCR reactions. PCRs were performed with pHS as a template and primers 1 and 2 (5′-GC**GGATCC**GTCGACTCTCTATAAAGCGATCG-3′ and 5′GGGAATTCAAGTTAGTAAATTATATATATAAT-3′, respectively) or 3 and 4 (5′-GC**GGTACC**GTCGACTCTCTATAAAGCGATCG-3′ and 5′-GGGAATTCTAGTTAGATAAATTAATAATATATAAG-3′, respectively). The 150-bp product synthesized with the first pair of primers contained *Bam*HI (boldface type, primer 1) and *Sal*I (underlined, primer 1) sites at one terminus and an *Eco*RI site (underlined, primer 2) at the other terminus. This product was cleaved with *Bam*HI and *Eco*RI and cloned into an appropriately digested pBSIIKS^{-Sal} vector. The cloned DNA was then digested with *Eco*RI and *Kpn*I DNA to serve as the recipient for the PCR product prepared with primers 3 and 4. This second PCR product contained *Kpn*I and *Sal*I sites at one terminus (boldface type and underlined, respectively, primer 3) and an *Eco*RI site at the other (underlined, primer 4). The final clone contained a 300-bp palindromic insert comprising the extended hairpin sequences and two copies of the flanking 87-bp unique region (see Fig. 5) from the vaccinia genome. The only divergence from the authentic genomic sequence was the insertion of 6 nt encoding an *Eco*RI site at the central axis.

The plasmid p65+tet contains a chimeric concatameric junction fragment in which the 130-bp viral concatameric junction is flanked on each side by 130 bp derived from the tetracyclin^r gene encoded by pBR322. PCR was performed on minichromosomes containing 65-bp viral telomeres (see below) using primers E (5′-AA**GGATCC**GTCGACTGTGGTCGCCATGATC-3′) and 2 (see above), or F (5′-AA**GGTACC**GTCGACTGTGGTCGCCATGATC-3′) and 4 (see above). The ≈200-bp product amplified with primers E and 2 contained an *Eco*RI site at one terminus (underlined, primer 2) and *Bam*HI (boldface type, primer E) and *Sal*I (underlined, primer E) sites at the other terminus. This product was cleaved with *Bam*HI and *Eco*RI and cloned into an appropriately digested pBSIIKS^{-Sal} vector. The cloned DNA was then digested with *Eco*RI and *Kpn*I to serve as the recipient for the PCR product amplified with primers F and 4. This second PCR product contained *Kpn*I and *Sal*I sites at one terminus (boldface type and underlined, primer F) and an *Eco*RI site at the other terminus (underlined, primer 4). The final clone contained a 410-bp palindromic insert with an *Eco*RI site at the central axis.

The plasmid p65+I1 contains a chimeric insert in which the 130-bp viral concatameric junction fragment is flanked on each side with 135 bp derived from the vaccinia I1 gene. A 510-bp *Nhe*I fragment was purified from plasmid pet14B/I1, a plasmid containing the vaccinia I1 gene (J. Ward and P.T., unpublished). The 510-bp segment was ligated to purified 65-bp vaccinia virus hairpins whose free terminus was generated by *Xba*I cleavage (ligation was performed in the presence of both *Nhe*I and *Xba*I), and the ligation products were used as the template for the following PCR. Amplification was performed with primers C (5′-GC**GGATCC**GTCGACTTCTGCAATATCTTCG-3′) and 2 (see above), or D (5′-GC**GGTACC**GTCGACTTCTGCAATATCTTCG-3′) and 4 (see above). The ≈210-bp product amplified with primers C and 2 contained an *Eco*RI site at one terminus (underlined, primer 2), and *Bam*HI and *Sal*I (boldface type and underlined, primer C) sites at the other terminus. This product was cleaved with *Bam*HI and *Eco*RI and cloned into an appropriately digested pBSIIKS^{-Sal} vector. The cloned DNA was then digested with *Eco*RI and *Kpn*I to serve as the recipient for the PCR product amplified with primers D and 4. This second PCR product contained *Kpn*I and *Sal*I sites at one terminus (boldface type and underlined, primer D) and an *Eco*RI site at the other terminus (underlined, primer 4). The final clone contained a 420-bp palindromic insert with an *Eco*RI site at the central axis.

Preparation of Viral Hairpins. The 3.7-kb viral terminal hairpin was isolated by cleavage of vaccinia virus genomic DNA with *Sal*I, fractionation by agarose gel electrophoresis, and purification on glass beads. The 200-bp viral hairpin was gel purified after pHS plasmid DNA was digested with *Sal*I, heated to 100°C for 3 min, and immediately snap-cooled on ice for 5 min The 150-bp, 65+tet, and 65+I1 hairpins were similarly generated by heating and snap-cooling the concatameric junction fragments (300, 410, and 420 bp, respectively) released by *Sal*I digestion of p150, p65+tet, and p65+I1. The 65-bp viral hairpin was similarly prepared by heating and snap-cooling a 130-bp concatameric junction fragment isolated after digestion of pSV9 with *Xba*I.

Preparation of Minichromosomes. Minichromosomes containing viral telomeres of different lengths (see Fig. 1) were constructed by one of two methods. For minichromosomes containing 3.7 kb, 200 bp, 150 bp, or 65 bp of viral sequence, or the 65+tet and 65+I1 chimeras, isolated hairpins were ligated to plasmid inserts. Each minichromosome was designed such that the hairpins and the central insert had compatible cohesive ends generated by distinct restriction endonucleases. Neither restriction site was regenerated in the desired ligation product (one hairpin at each terminus of the linear plasmid), and therefore ligations were performed in the presence of both restriction enzymes to drive the reaction to the desired product and away from other possible ligations (hairpin/hairpin, insert/insert). The 3.7-kb, 200-bp, 150-bp, 65+tet, and 65+I1 hairpins contained termini generated by *Sal*I cleavage and were ligated to pBSIIKS^{-SalI} DNA linearized with *Xho*I. The 65-bp hairpin was generated by cleavage with *Xba*I and was ligated to pBR322 DNA linearized with *Nhe*I. Initially, ligation products were recovered by ethanol precipitation and treated with exonuclease III to eliminate nicked DNA. Nuclease treatment was suspended upon determination that essentially all of the minichromosomes in ligated preparations were covalently closed. Minichromosomes were then purified on glass beads with or without prior fractionation on agarose gels.

Minichromosomes bearing 1.3 kb of viral telomeric sequences were prepared by ligation of snap-cooled, *Eco*RI-digested pSV9. *Eco*RI digestion cleaves this dimeric plasmid into two fragments that renature into 4- and 1.3-kb hairpins. Ligation creates the desired product (5.3-kb minichromosome) as well as 8-kb and 2.6-kb minichromosomes that can be resolved electrophoretically.

A control minichromosome, in which pBSIIKS DNA was ligated to synthetic, 26-nt hairpins was also prepared. The synthetic hairpin was synthesized on an Applied Biosystems oligonucleotide synthesizer and had the following sequence: 5′-TCGACGGTACCGTTTTGCGGTACCG-3′. Heating, snap-cooling, and terminal phosphorylation of this oligonucleotide left termini that were compatible with ligation to *Xho*I-cleaved pBSIIKS^{-Sal} DNA.

To quantitate the concentration of minichromosome preparations, DNAs were transferred to nitrocellulose and hybridized to a radiolabeled probe derived from the β-lactamase gene. In addition to quantitating intact minichromosomes, Southern blot analysis was performed on minichromosomes in which the plasmid inserts had been released by cleavage with appropriate restriction endonucleases prior to electrophoretic fractionation. The β-lactamase genes were therefore on DNA fragments of similar size, ensuring uniform capillary transfer to the filter.

***In Vivo* Replication of Minichromosomes.** Mouse L cells were split onto 60-mm-diameter culture dishes 24 h before infection. Confluent L cells were then infected with vaccinia virus at a multiplicity of infection of 3 plaque-forming units/cell. After 30 min of adsorption, the DNA substrate and 45 μl of lipofectin were added to 600 μl of DMEM and applied according to the instructions of the manufacturer. At 6.5 h postinfection (hpi), the lipofection mixture was removed and cells were fed with fresh DMEM supplemented with 5% fetal calf serum (GIBCO/BRL). Infected cells were harvested at 24 hpi except where indicated (see Fig. 3). Cells were washed once with phosphate-buffered saline (140 mM NaCl/2 mM KCl/10 mM Na_2HPO_4/1 mM KH_2PO_4, pH 7.4) and resuspended in 135 μl of hypotonic buffer (10 mM Tris·HCl, pH 8.0/10 mM KCl/5 mM EDTA). After 5 min on ice, Triton X-100 and 2-mercaptoethanol were added to 1% and 40 mM, respectively, and incubation was continued for an additional 10 min with occasional mixing. Nuclei were removed by sedimentation at 2700 rpm for 10 min in a microfuge. Cytosolic lysates were then supplemented with 90 μg of proteinase K per ml, 0.6% SDS, and 176 mM NaCl and gently mixed at 37°C for 2 h. After organic extraction, nucleic acids were recovered by ethanol precipitation. The DNA pellet was resuspended in 20 μl of 10 mM Tris (pH 7.4) and 1 mM EDTA.

RESULTS

The vaccinia virus genome is a double-stranded, linear molecule with covalently closed hairpin termini (18). It seems likely that both the topology and the sequence of the telomeric regions play a role in ensuring efficient DNA replication. We therefore designed minichromosome templates that retained the topology of the authentic viral genome but varied in the extent of the telomeric sequences that they included (Fig. 1) Basically, all minichromosomes were linear, covalently closed molecules that contained two equivalent telomeres flanking central plasmid-derived sequences. The plasmid sequences, isolated from bacteria, were methylated and hence sensitive to cleavage by *Dpn*I; were these sequences to undergo replication after introduction into vaccinia-infected eucaryotic cells, they would become resistant to *Dpn*I cleavage.

The 3.7-kb Vaccinia Virus Telomeric DNA Contains cis-Elements That Enhance Template DNA Replication. As a first test of our assay, cells were infected with vaccinia virus and then incubated with lipofection mixtures containing linear or supercoiled plasmid DNA (Fig. 1, substrates 7 and 8), minichromosomes containing 3.7 kb of viral telomeric sequences at each end (Fig. 1, substrate 1), or control minichromosomes in which the central plasmid region was flanked by synthetic, 26-nt hairpins bearing no resemblance to viral telomeres (Fig. 1, substrate 6). Cytoplasmic DNA was recovered at 24 hpi and the levels of *Dpn*I-resistant plasmid sequences were assessed. As is evident in Fig. 2 *Left*, the minichromosomes containing the 3.7-kb viral telomeres (lane 6) replicated the most efficiently. The minichromosomes containing only a synthetic hairpin (lane 5) replicated poorly if at all, and linear templates with open termini were apparently unstable (lane 4), since even the input, *Dpn*I-sensitive species (bracketed) were not detected. Although the supercoiled DNA substrate (lane 3) was expected to replicate to some extent, its replication was nearly undetectable and was obviously far less efficient than that of the minichromosome template. As one way to compare the relative efficiencies of replication, increasing amounts of supercoiled substrates were applied to infected cells (Fig. 2 *Right*); at least 10-times as much supercoiled DNA as minichromosome DNA had to be applied to generate the same level of *Dpn*I-resistant product at 24 hpi.

Replication of the Minichromosomes Containing 3.7-kb Viral Telomeres Occurs from 6–24 hpi and Depends Upon Viral Replication Machinery. To determine when minichromosome replication was taking place, cells were infected at a multiplicity of infection of 3 and then lipofected with minichromosomes containing 3.7-kb viral telomeres. Cytoplasmic DNA was harvested at 1, 3, 6, 12, and 24 hpi, and the levels of total (undigested) and replicated (*Dpn*I-resistant) plasmid sequences were visualized by Southern blot hybridization (Fig. 3 *C–E*) and quantitated by densitometry. From direct comparison with known quantities of DNA (Fig. 3*A*), we estimated that ≈0.5% of the DNA introduced by lipofection was stably cell-associated after 1 h (70 amol retained, 13 fmol applied). Cell-associated levels of minichromosomes declined 2-fold by 3 hpi and remained at that level until 6 hpi (Fig. 3*C*). *Dpn*I-resistant material was first seen at 6 hpi (Fig. 3*E*) (3% of the total minichromosomes) and increased dramatically (>40-fold) in abundance until 24 hpi (Fig. 3 *D* and *E*). At this time point, ≈60% of the intracellular minichromosomes were recovered in a replicated, *Dpn*I-resistant form (compare 24 hpi, Fig. 3 *C* and *D*). The ability to distinguish replicated from unreplicated DNA was therefore of significant value, since even with this most efficiently replicating substrate, there was only a 2-fold increase in the total amount of cell-associated minichromosome DNA between 6 and 24 hpi.

To further demonstrate that replication of minichromosomes was accomplished by the vaccinia replication machinery, replication was also assessed in uninfected cells and in cells infected permissively (32°C) and nonpermissively (39.5°C) with *ts*17 and

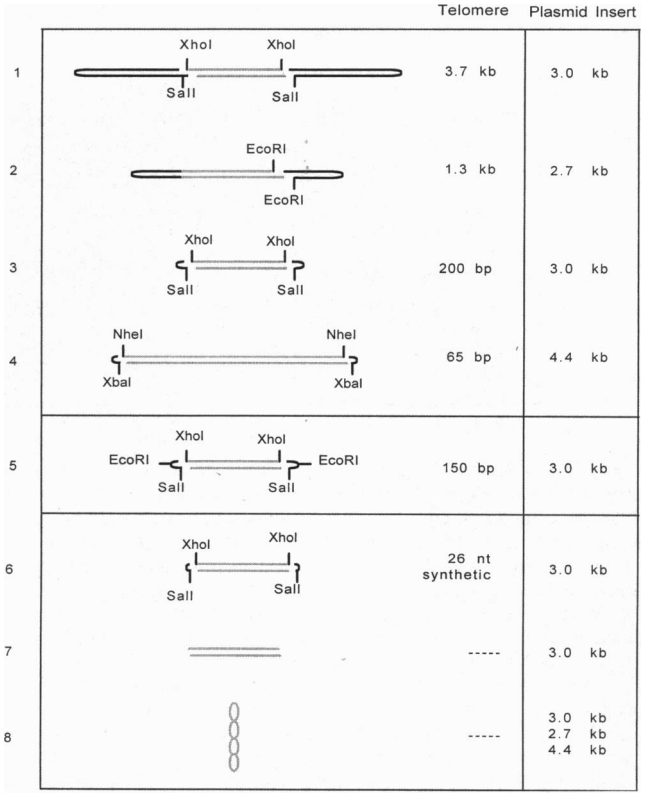

FIG. 1. Minichromosome and control substrates used to define the cis-acting sequences required for template replication within vaccinia-infected cells. Minichromosome substrates 1–6 contained a central plasmid region (grey lines) and progressively shorter hairpin telomeres (black lines). The plasmid sequences were derived either from pBSI-IKS (substrates 1, 3, 5, and 6), pUC 13 (substrate 2), or pBR322 (substrate 4). Telomeres were comprised of vaccinia genomic sequences (substrates 1–5) or a synthetic oligonucleotide sequence (substrate 6). The restriction endonuclease sites at the junctions of the telomere and plasmid sequences (and at the tip of the hairpin for substrate 5) are indicated, and the sizes of each component are shown to the right. A map of the sequence elements contained within the various telomeres is shown in Fig. 5. Linearized pBSIIKS (substrate 7) and supercoiled pBSIIKS, pUC19, or pBR322 (substrate 8) were also used as controls.

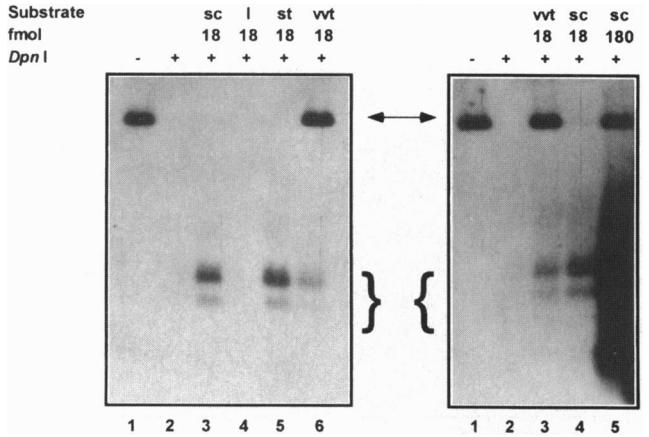

FIG. 2. Vaccinia virus 3.7-kb telomeres enhance replication of exogenous substrates. (*Left*) DNA substrates (18 fmol) were introduced into mouse L cells infected with vaccinia virus (multiplicity of infection of 3); at 24 hpi, DNA was harvested and treated with *Bss*HII to release the plasmid insert from minichromosome substrates or linearize supercoiled substrates and *Dpn*I to digest unreplicated input DNA. Replication products were then fractionated by agarose gel electrophoresis and analyzed by Southern blot hybridization. Lanes 1 and 2 contain *Bss*HII-linearized pBSIIKS DNA, before and after *Dpn*I treatment, as markers. Lanes 3–6 contain the *Dpn*I-treated samples prepared from DNA harvested from cells exposed to supercoiled pBSIIKS (sc), linearized pBSIIKS (l), minichromosomes containing a 26-nt synthetic telomere (st), or minichromosomes containing the 3.7-kb viral telomere (vvt) (substrates 8, 7, 6, and 1 of Fig. 1, respectively). (*Right*) Lanes 1 and 2 contain the same reference samples as *Left*. Lanes 3–5 contain *Dpn*I-treated samples prepared from DNA harvested from infected cells exposed to 18 fmol of the minichromosomes containing the 3.7-kb viral telomere (vvt) or 18 or 180 fmol of supercoiled pBSIIKS (sc), respectively. *Dpn*I-resistant material is indicated by the double arrow; products generated by *Dpn*I-digestion are bracketed.

*ts*42, viral mutants that fail to direct DNA synthesis at the nonpermissive temperature (14–16). No minichromosome replication was seen in uninfected cells or during nonpermissive infections with either *ts* mutant (not shown).

Minichromosomes Containing 200-bp Vaccinia Virus Telomeres Retain Maximal Replication Activity. Having established that minichromosomes bearing 3.7-kb viral telomeres replicated far more efficiently than control minichromosomes or supercoiled substrates, we set out to further define the cis-acting elements within these terminal sequences. To achieve this goal, a series of minichromosomes bearing progressively shorter hairpin termini were constructed (Fig. 1, substrates 2–4). Based on the availability of convenient restriction enzyme sites and the known structure of the viral telomeres, substrates containing 3.7 kb, 1.3 kb, 200 bp, and 65 bp were constructed.

All of the minichromosomes and control substrates were quantitated by Southern blot hybridization and phosphoimage analysis (Fig. 4*A Upper*). Plasmid sequences were released from the substrates by restriction endonuclease digestion before electrophoresis, and blots were hybridized with sequences derived from the *E. coli* β-lactamase gene. For each substrate, 10 fmol was introduced into infected cells by lipofection, and cytoplasmic DNA was harvested at 24 hpi. The replicated, *Dpn*I-resistant products are shown in Fig. 4*A Lower*. These data reveal that the 3.7-kb, 1.3-kb, and 200-bp viral telomere enhanced template DNA replication to the same extent. In contrast, the other substrates tested replicated poorly. Data from three independent experiments are shown schematically in Fig. 4*B*. All data were normalized to the results obtained with the control minichromosome bearing the synthetic, 26-nt hairpin. The three supercoiled DNAs tested replicated about 10-times more efficiently than this control substrate; minichromosomes with the 65-nt hairpin replicate approximately 6-fold more efficiently. Most importantly,

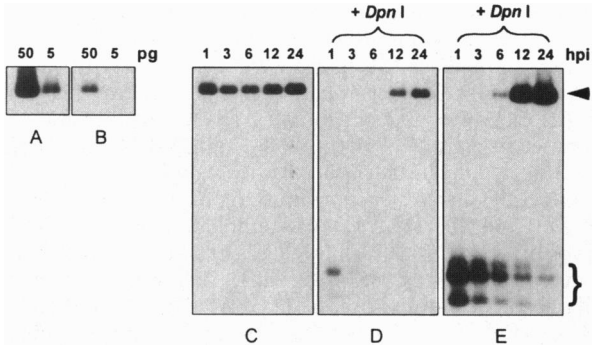

FIG. 3. Efficiency and time course of minichromosome replication. Minichromosomes containing the 3.7-kb viral telomere (13 fmol) were introduced into infected cells by lipofection; cytoplasmic DNA was harvested at 1, 3, 6, 12, and 24 hpi and subjected to electrophoretic fractionation and Southern blot hybridization before (*C*) or after (*D* and *E*) digestion with *Dpn*I. (*E*) An 8-fold longer autoradiographic exposure of *D*. (*A* and *B*) Two different autoradiographic exposures (8-fold difference in duration) of 50 and 5 pg of linearized pBSIIKS (25 and 2.5 amol, respectively). (*A* and *E*) Equivalent exposures; (*B* and *D*) equivalent exposures. The *Dpn*I-resistant plasmid insert is indicated with an arrowhead; *Dpn*I-digestion products are bracketed.

the 200-bp, 1.3-kb, or 3.7-kb telomeres increase the replication efficiency by 120- to 135-fold and do not appear to be significantly different from each other. The 200-bp telomere appears to contain all of the cis-elements required for maximal minichromosome replication.

Sequences Within the 87-bp Unique Region Proximal to the Terminal Hairpin Are Important for Maximal Replication Activity. The 200-bp telomeres that are sufficient to confer maximal replication efficiency on minichromosome templates contain the 104-nt A+T-rich hairpin, an 87-bp unique region, and the majority of the first of 31 copies of the 70-bp repeat motif (Fig. 5). The observation that minichromosomes containing a large number of 70-bp repeat elements (1.3- and 3.7-kb telomeres) were not superior templates for replication suggested that these repeat elements were unnecessary for template recognition and/or utilization. To explore this point more carefully, we utilized PCR technology to assemble minichromosomes containing 150 bp of viral sequence comprising only the hairpin and 87-bp unique regions (substrate 5, Fig. 1). The relative replication efficiency of these minichromosomes was 95-fold greater than the control substrate in experiments in which the 200-bp telomeres conferred a relative replication efficiency of 140 (Fig. 6). Clearly, the 150-bp telomeres retain most, but not all, of the elements required for optimal template utilization. Whether the modest decrease in replication efficiency seen with substrates containing the 150-bp telomeres (as compared with those containing 200-bp telomeres) reflects the loss of the distal repeat element or the insertion of 6 nt at the apex of the hairpin has not been determined.

Although our data are fully compatible with the hypothesis that optimal replication requires specific sequence elements contained within the 200-bp telomere but not within the 65-bp telomere, it is also plausible that the telomeres must simply be of a minimal size to function properly. To address this question, we constructed minichromosomes containing chimeric telomeres of 200 bp. In these telomeres, the terminal 65-bp sequences were fused to ectopic sequences derived either from the vaccinia virus I1 gene (65+I1) or from the bacterial tetracycline^r gene (65+tet). The relative replication efficiency conferred by these chimeric telomeres is shown in Fig. 6. Clearly, they were no more effective than the 65-bp telomere in directing minichromosome replication, proving that telomere function is sequence-, and not size-, specific, and that the 87-bp region plays an important role in template utilization.

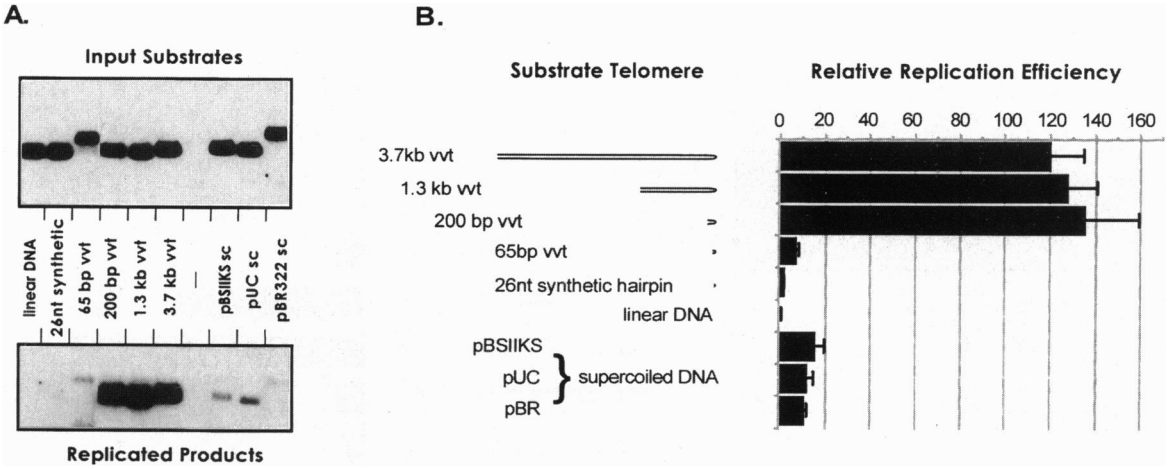

FIG. 4. Quantitation and comparison of the replication efficiencies of the substrates. (*A*) Vaccinia-infected cells were exposed to the following replication substrates: linearized pBSIIKS plasmid (Fig. 1, substrate 7), minichromosomes containing the 26-nt synthetic telomere or viral telomeres of 65, 200, 1300, or 3700 bp in length (Fig. 1, substrates 6, 4, 3, 2, and 1, respectively), or supercoiled pBSIIKS, puc19, or pBR322 plasmids (Fig. 1, substrate 8). (*Upper*) Approximately 1 fmol of each substrate was subjected to electrophoretic fractionation and Southern blot hybridization after release of the plasmid insert by restriction endonuclease digestion, and the equivalence of the substrate levels was confirmed by phosphoimage analysis. (*Lower*) For each substrate, 10 fmol was introduced into vaccinia virus-infected cells by lipofection; cytoplasmic DNA was harvested at 24 hpi, digested with the appropriate restriction endonucleases to release the plasmid insert, and treated with *Dpn*I. Replication products were then subjected to electrophoretic fractionation and Southern blot hybridization. (*B*) The replication efficiency of each substrate was quantitated and compared with the replication efficiency of the minichromosomes containing the 26-nt synthetic hairpin. Data were quantitated by phosphoimage analysis, and the average value obtained from three experiments is shown with standard errors indicated.

Fig. 6*A* illustrates an additional point regarding the conformation of the replicated minichromosomes. The electrophoretic mobility of the input minichromosomes was compared with that of the *Dpn*I-resistant replication products. The replicated minichromosomes bearing the 150-bp and 200-bp telomeres clearly comigrate with the relevant input molecules, indicating that they represent resolved, monomeric genomes.

DISCUSSION

Establishment of an Assay That Demonstrates Sequence and/or Structural Requirements for Vaccinia Virus DNA Replication. In this report we have provided definitive evidence for template specificity in vaccinia virus DNA replication. Control templates with synthetic hairpin termini were stable but failed to replicate, whereas minichromosomes containing at least the terminal 150 bp of the viral chromosome replicated 95- to 140-fold more efficiently. Remarkably consistent data were obtained with independent preparations of minichromosomes. The terminal 65 bp conferred only minimal replication efficiency (6-fold above the control). The values obtained with the optimal minichromosomes are far higher than the nonspecific replication observed for supercoiled plasmid templates (10-fold above control minichromosomes). It was this low level of nonspecific plasmid replication that had

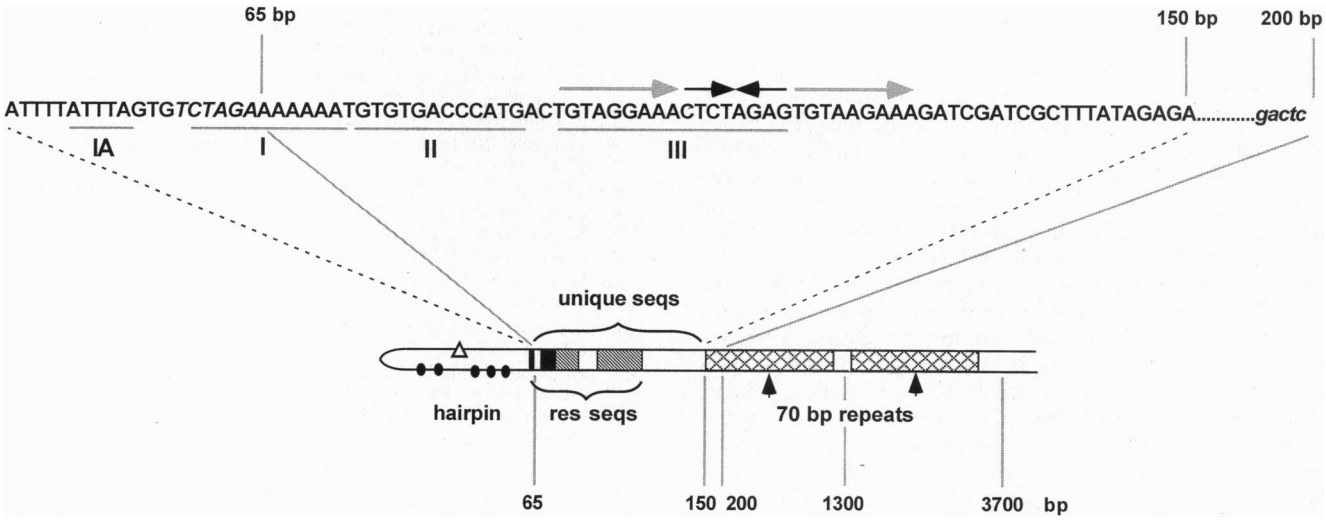

FIG. 5. Map of the sequences contained within the terminal 3.7 kb of the viral genome. A schematic representation of the telomeric 3.7 kb of the viral genome is shown in the middle of the figure. Salient features, moving inwards from the terminus, include the hairpin, unique sequences (seqs) including the sequences required for concatamer resolution (res seqs), and two blocks of 70-bp repeats (cross-hatched). The filled ovals and open triangle shown in the hairpin depict the extrahelical bases found within this exclusively A+T-rich 104-nt region. The black and shaded boxes within the resolution sequences depict boxes IA, I and II, III, respectively. The grey lines extending downward from this schematic illustration mark the internal boundaries of the 65-, 150-, 200-, 1300-, and 3700-bp telomeres. The sequence of the 87-bp unique region is expanded at the top of the figure (dashed lines); resolution boxes IA, I, II, and III are underlined, and the internal boundaries of the 65-, 150-, and 200-bp telomeres are shown (grey lines). The restriction sites that define these boundaries are shown in italics. The black and grey arrows above the sequence indicate the arrangement of an inverted repeat flanked by two direct repeats, respectively.

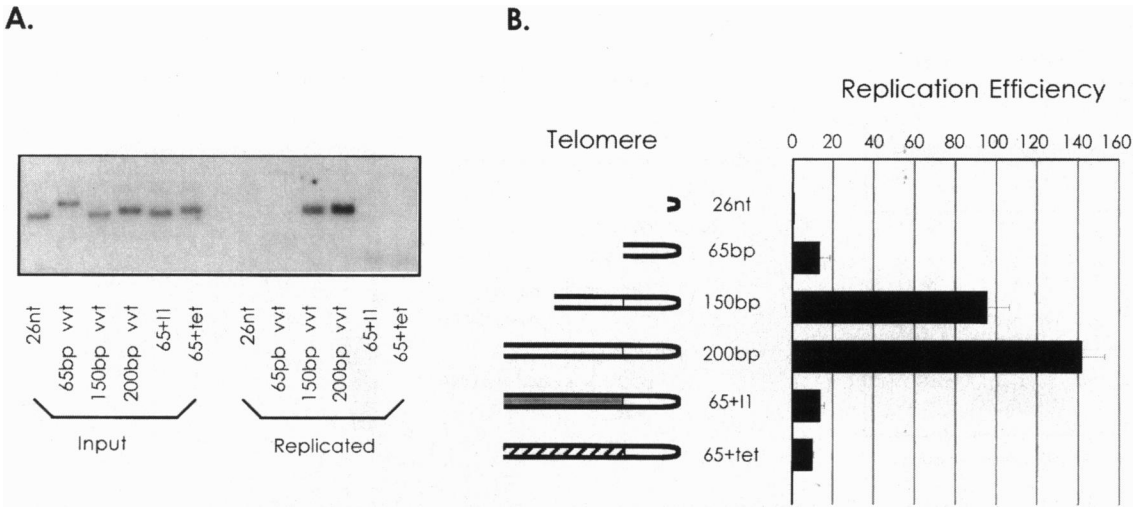

FIG. 6. Analysis of the role of the 87-bp unique region in minichromosome replication. (*A*) Vaccinia-infected cells were exposed to 10 fmol of minichromosomes containing the 26-nt, 65-bp, 150-bp, or 200-bp telomeres (substrates 6, 4, 5, and 3 in Fig. 1, respectively) or chimeric telomeres containing 65 bp from the viral genome and 135 bp from the viral I1 gene (65+I1) or the bacterial tetracycline^r gene (65+tet). At 24 hpi, cytoplasmic DNA was recovered and digested with *Dpn*I. Replicated DNA was resolved by electrophoresis and visualized by Southern hybridization; these samples are shown in the lanes marked as "replicated". For each of the input substrates, 10 amol is shown in the lanes marked as "input." (*B*) Replication efficiency of each substrate was quantitated and compared with the replication efficiency of the minichromosomes containing the 26-nt synthetic hairpin. Data were quantitated by phosphoimage analysis, and the average value obtained from three experiments is shown with standard errors indicated.

previously been noted by several investigators and had implied that there might be no specificity in recognition and utilization of templates by the vaccinia replication machinery (10). Our data indicate that there is indeed significant specificity.

Confidence that the replication of these exogenous templates relies on the viral enzymatic machinery comes from the inability of uninfected cells or cells infected with temperature-sensitive DNA$^-$ mutants to direct template replication, as well as by the observed time course of replication. Several lines of evidence suggest that the replication seen occurs by the same mechanism employed during viral replication. We have verified that the replication products are covalently closed linear molecules by examining their electrophoretic behavior under both native and denaturing conditions (not shown). The templates that replicate efficiently contain the concatamer resolution boxes IA, I, II, and III (19), and therefore it is not surprising that only mature, monomeric replication products are recovered. Minichromosomes containing only 65 bp of telomeric sequence contain only box IA and a portion of resolution box I, and consequently dimers and trimers are among the low yield of replication products. Our confidence that the synthesis seen is *bona fide* replication, rather than repair, is bolstered by the observation that there is a net increase in the level of minichromosome DNA between 6 hpi, when replication was first seen, and 24 hpi.

Analysis of the Sequence/Structure Content of the 87-bp Viral Telomeric Region. Two main elements are contained within the telomeric sequences required for template replication: the 104-nt A+T-rich hairpin with its 12 extrahelical bases and the 87-bp unique region (see Fig. 5). Our studies indicate that the terminal 65 bp of the viral genome, while encompassing the entire hairpin, are not sufficient to confer template replication. Although a hairpin *per se* is required to ensure template stability, we do not know whether sequences and/or structures within the viral hairpin are specifically required.

Comparison of the replication efficiences of substrates containing 150- or 200-bp viral telomeres with those containing chimeric telomeres in which the terminal 65 bp are juxtaposed with ectopic viral or nonviral sequences indicates that the 87-bp region is specifically required for efficient replication. This 87-bp region is highly conserved among all poxviruses and contains concatamer resolution elements IA, I, II, and III (19). The region also contains a strong late promoter (20, 21) as well as an inverted repeat flanked by two direct repeats (TGTAGGAAACTCTAGAGTGTAAGAAA, overlined in Fig. 5), a motif that could sustain protein/DNA interactions. Our findings provide a firm basis for the finer dissection of the cis-acting sequences required for the replication of the vaccinia genome and the trans-acting proteins with which they interact. These studies should be provocative in light of the diversity of mechanisms by which the initiation of DNA replication is accomplished.

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