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Efficient Resolution of Replicated Poxvirus Telomeres to Native Hairpin Structures Requires Two Inverted Symmetrical Copies of a Core Target DNA Sequence

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The terminal hairpin sequences of the linear double-stranded DNA genome of the leporipoxvirus Shope fibroma virus (SFV) has been cloned in *Saccharomyces cerevisiae* and in recombination-deficient *Escherichia coli* as a palindromic insert within circular plasmid vectors. This sequence configuration is equivalent to the inverted repeat structure detected as a telomeric replicative intermediate during poxvirus replication in vivo. Previously, it has been shown that when circular plasmids containing this palindromic insert were transfected into SFV-infected cells, efficient replication and resolution generated linear minichromosomes with bona fide viral hairpin termini (A. M. DeLange, M. Reddy, D. Scraba, C. Upton, and G. McFadden, J. Virol. 59:249–259, 1986). To localize the minimal target DNA sequence required for efficient resolution, a series of staggered unidirectional deletions were constructed at both ends of the inverted repeat. Analyses of the resolution efficiencies of the various clones indicate that up to 240 base pairs (bp) centered at the symmetry axis were required for maximal resolution to minichromosomes. To investigate the role of the AT-rich central axis sequences, which in SFV include 8 nonpalindromic bp, a unique *Afl*II site at the symmetry axis was exploited. Bidirectional deletions extending from this *Afl*II site and insertions of synthetic oligonucleotides into one of the deletion derivatives were constructed and tested in vivo. The efficiency with which these plasmids resolved to linear minichromosomes with hairpin termini has enabled us to define the minimal target DNA sequence as two inverted copies of an identical DNA sequence between 58 and 76 bp in length. The nonpalindromic nucleotides, which, after resolution, constitute the extrahelical residues characteristic of native poxviral telomeres, were not required for resolution. The close resemblance of the SFV core target sequence to the analogous region from the orthopoxvirus vaccinia virus is consistent with a conserved mechanism for poxviral telomere resolution.

The large majority of eucaryotic cellular and viral genomes exist as linear double-stranded DNA molecules whose termini, though sometimes heterogeneous in length, are highly stable structures (for reviews, see references 4, 5, and 7). In contrast, broken DNA ends generated intracellularly, e.g., after X-ray treatment, are unstable and fuse with other nontelomeric ends. Since all known DNA polymerases require a primer with a 3′-hydroxyl end, special mechanisms have been invoked to explain the accurate replication and maintenance of genomic termini. Cavalier-Smith (9) proposed that the ends of linear eucaryotic chromosomes are palindromes which can form base-paired hairpin structures capable of acting as primers. A variation of this model proposed that the chromosomal ends exist as self-complementary hairpin structures (2). Naturally occurring hairpin termini have been observed in *Paramecium* mitochondrial DNA (24), *Tetrahymena* ribosomal DNA (6), linear *Saccharomyces cerevisiae* plasmids (16), parvovirus DNA (3, 8), and poxvirus DNA (1, 12, 13).

The genome of the prototype orthopoxvirus, vaccinia virus, consists of a single 185-kilobase linear double-stranded DNA molecule with terminal inverted repeats in excess of 10 kilobases and hairpin ends that connect the two complementary strands into one continuous polynucleotide chain (reviewed in references 18, 20, and 21). The terminal AT-rich 104 nucleotides of the viral hairpin are not completely base-paired and exist in two alternate forms that are inverted and complementary to each other (1). The general features of this hairpin terminus have been conserved in the tumorigenic leporipoxvirus Shope fibroma virus (SFV), whose terminal 64 nucleotides are AT-rich and exist in two alternate ("flip" and "flop") conformations (12).

The hairpin termini of vaccinia virus and SFV have been cloned as imperfect inverted repeats with the original hairpin ends at the axis of symmetry (11, 12, 19, 23, 26). This inverted repeat arrangement has also been observed during DNA replication of several poxviruses (10, 21, 22). To evaluate the utility of these cloned viral telomeres as substrates for enzymes that convert the inverted repeat forms to genuine hairpin termini, we developed an in vivo transfection assay that allows replication of exogenous plasmid DNA in poxvirus-infected cells (10). Replication of plasmid DNA transfected into poxvirus-infected cells generates long concatemers of the plasmid DNA in a fashion that does not depend on the presence of a *cis*-acting DNA replication origin sequence (10). When plasmids containing the cloned "inverted repeat" configuration of SFV or vaccinia virus termini were transfected in cells that had previously been infected with homologous virus, replication and resolution of the viral inverted repeats generated linear monomer and multimer minichromosomes with viral hairpins at both ends (12, 19). The amount of replicated, and therefore resolved, plasmid DNA is routinely 1 to 2 orders of magnitude larger in rabbit cells infected with leporipoxviruses, such as SFV or myxoma virus, than in cells infected with orthopoxviruses, such as vaccinia virus. The consistently high levels of replicated minichromosomes obtained in leporipoxvirus-

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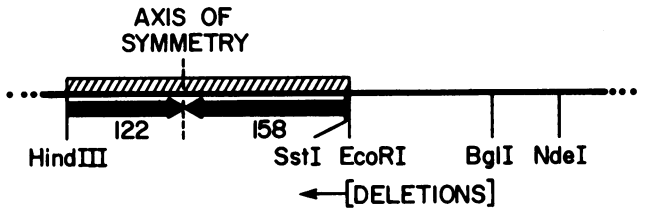

FIG. 1. Construction scheme for reverse unidirectional deletions of the SFV telomeric sequences in pSD19. Plasmid pSD19 was derived from pSAlB-56A by the deletion of 42 bp from the left end of the SFV inverted repeat insert and contains 280 bp of the viral telomeric inverted repeat, 122 bp at the left side of the axis of symmetry and 158 bp at the right side. The viral DNA sequence is indicated by black arrows. Unidirectional deletions from the right end were generated by digestion of pSD19 with *Eco*RI and *Bgl*I, followed by treatment for various periods with exonuclease III, which digests from the *Eco*RI cleavage site but not from the *Bgl*I cleavage site, as described in Materials and Methods.

infected rabbit cells greatly facilitate analysis of telomere resolution.

In a preliminary attempt to define the outer limits of the target DNA sequences that are required for resolution of the inverted repeat arrangements of the SFV and vaccinia telomeres, we constructed and tested unidirectional deletions starting from one end of respective inverted repeats (12). It was found that within the 240- to 280-base-pair (bp) regions of SFV and vaccinia virus that are sufficient for resolution lie several sequence elements that are either fully conserved [the 11-bp region I: $(T)_7CTAG$] or whose order of purines and pyrimidines is almost perfectly conserved (13 bp for region II and 17 or 18 bp for region III). It may therefore not be completely unexpected that the SFV telomere was resolved, although with a reduced efficiency, by vaccinia virus and conversely that the vaccinia virus telomere was resolved by SFV (12). These observations suggest that the telomeres of these two divergent poxviruses are both structurally and functionally conserved. In this communication, we define in more detail both the external and internal boundaries of the target DNA sequence required for resolution of the SFV inverted repeat intermediate to daughter hairpin termini.

MATERIALS AND METHODS

Plasmids. Plasmid pSD19 used in the plasmid constructions described in this paper contains 280 bp from the SFV telomeric palindrome cloned into pUC13 and is a deletion derivative of pSAlB-56A (described in reference 12). Plasmid pAT34 contains an insert of 34 alternating A and T residues and was obtained from D. Pulleyblank (15). Plasmid pAL114 (25) contains a perfect palindrome of 114 bp and was obtained from D. Evans. The construction of unidirectional deletions in pSD19 is illustrated in Fig. 1. pSD19 was digested partially with *Bgl*I, and the full-length linear molecules were purified by preparative agarose gel electrophoresis and then digested with *Eco*RI. Staggered unidirectional deletions of the resulting 2.8-kilobase linear species were created by using exonuclease III plus exonuclease VII and propagated in the recombination-deficient *Escherichia coli* DB1256 (*recA recB recC sbcB*), as described by DeLange et al. (12). Size screening of the resultant clones was done with *Hin*dIII plus *Nde*I (Fig. 1), and candidate deletions were verified by Sanger dideoxy sequencing. Bidirectional central axis deletions of pSD19 were created from the unique *Afl*II site (CTTAAG) at the symmetry axis of the SFV telomere (see Fig. 3 of reference 12) as follows. Since a fraction of the purified plasmid DNA has been extruded into the cruciform configuration and is therefore resistant to *Afl*II, pSD19 was partially relaxed by reaction with calf thymus topoisomerase I for 16 h at 37°C in 0.2 M KCl–2 mM EDTA–10 mM Tris (pH 8)–100 μg of gelatin per ml–0.5 μl topoisomerase I (gift of A. R. Morgan). The resulting DNA was extracted with phenol-chloroform and digested with *Afl*II (Amersham Corp.), and the digested linear molecules were separated from unreacted circular molecules by preparative agarose gel electrophoresis in the presence of 0.5 μg of ethidium bromide per ml. The resulting *Afl*II-linearized pSD19 was then digested with exonuclease III plus exonuclease VII as described above. Bidirectional deletions resulting from this procedure were propagated in *E. coli* DB1256 and screened for insert sizes with *Eco*RI plus *Hin*dIII. Candidate clones were sequenced, and the following isolates were chosen for analysis: pD5-16 (Δ27), pD6-3 (Δ71), pD5-10 (Δ108), pD5-1 (Δ180), and pD6-20 (Δ169). Clone pD5-10 was a perfectly symmetrical deletion and resulted in a 130-bp palindromic insert of SFV near-terminal sequences with a unique *Hpa*I site (GTTAAC) at its central axis. To create central-axis additions to pD5-10, the plasmid was relaxed as described above for pSD19 with topoisomerase I and digested with *Hpa*I (which generates flush ends). The linear molecules were isolated by preparative gel electrophoresis in the presence of ethidium bromide and blunt-end ligated with a variety of double-stranded oligonucleotide linkers. Three types of oligonucleotides were inserted in central-axis deletion plasmid pD5-10: (i) *Eco*RI-*Hha*I nonpalindromic adaptor 8-mer (5′-AATTCGCG-3′); (ii) *Bam*HI palindromic linker 8-mer (5′-CGGATCCG-3′); and (iii) the palindromic oligonucleotide 22-mer (5′-ATAAATTACTTAAGTAATTTAT-3′). As before, clones were propagated in *E. coli* DB1256. The following clones are described in terms of their inserts: pSA5/10-A27 (one copy of the *Eco*RI-*Hha*I adaptor); pSA5/10-B32, -B17, -B24, and -B27 (one, two, three, and five copies, respectively, of *Bam*HI linkers); and pSAD-2 (one copy of the palindromic 22-mer).

DNA sequencing and transfection assay. All deletion endpoints were determined by DNA sequencing, with plasmid DNA as the template in dideoxynucleotide chain termination reactions as previously described (12). For the reverse deletions of pSD19 (Fig. 1), the primer used was an oligonucleotide synthesized by the Regional DNA Synthesis Laboratory, University of Calgary, Alberta, Canada (5′-GCACAGATGCGTAAGGAGAA-3′) and designed to flank the *Bgl*I site of pUC13 so as to prime in the orientation of the insert deletions. The number of *Bam*HI linker inserts in the pSA5/10-B series were determined by acrylamide gel electrophoresis of DNA fragments generated by complete *Hin*dIII and partial *Bam*HI digestion. The in vivo resolution assay involving transfection of calcium phosphate-precipitated plasmid DNA into SFV-infected SIRC cells has been described previously (10, 12). For all experiments, 0.25 μg of DNA was added per lane, and autoradiogram exposures were for 1 to 2 days.

RESULTS

Unidirectional deletions of the SFV telomere. Plasmid pSAlB-56A contains the hairpin terminus of SFV as a 322-bp imperfect inverted repeat with 8 nonpalindromic bp near its axis of symmetry (12). The viral DNA sequence in this plasmid is, when transfected into SFV-infected SIRC cells,

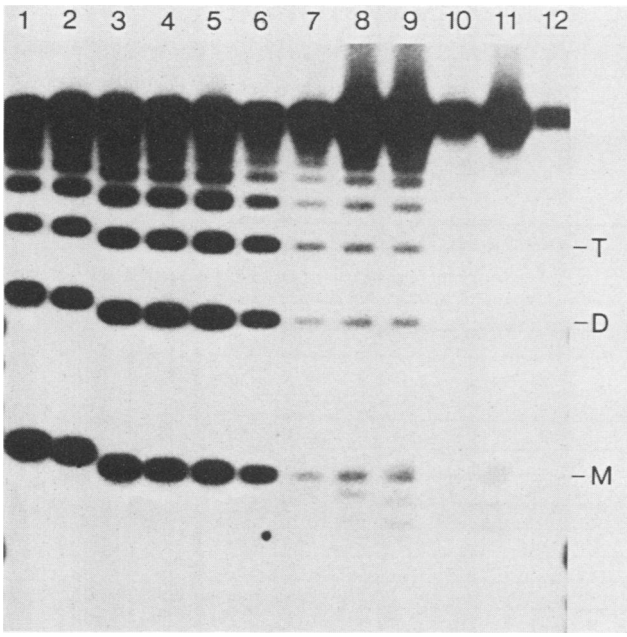

FIG. 2. Resolution of plasmid pSD19 to linear minichromosomes and comparison with the reverse unidirectional deletions derived from pSD19. Calcium phosphate-precipitated plasmid DNA (50 ng) was transfected into monolayers of SIRC cells that had been infected with SFV at a multiplicity of infection of 1. The DNA was extracted after 24 h, digested with *Dpn*I to cleave any nonreplicated input plasmid DNA, electrophoresed in 0.7% agarose, blotted, and hybridized with ^{32}P-labeled vector pUC13 DNA. Monomer (M), dimer (D), trimer (T), and higher oligomeric linear minichromosomes are shown. The deletion plasmids are diagrammed in Fig. 3. Lanes: 1, pSAlB-56A; 2, pSD19; 3, pD20; 4, pD4; 5, pD3; 6, pD21; 7, pD13; 8, pD24; 9, pD8; 10, pD9; 11, pD11; and 12, pD1. Longer exposures of this blot (not shown) displayed faint monomer and multimer resolved minichromosomes in lanes 10 (pD9) and 11 (pD11), but none in lane 12 (pD1).

recognized by *trans*-acting virus-induced proteins and resolved to linear minichromosomes with viral hairpin termini. By making use of unidirectional deletions from one end of this inverted repeat, we were able to identify one boundary of the *cis*-acting DNA sequence required for resolution (12). Between this boundary and the axis of symmetry, we located a sequence domain, which includes three regions designated I, II, and III, that is highly homologous to the analogous domain defined for an orthopoxvirus, vaccinia virus. The two inverted copies of this domain are separated by an AT-rich internal sequence which includes the nonpalindromic base pairs and flanks the central axis of symmetry of the inverted repeat. To define the boundary at the opposite side of the axis of symmetry, we chose plasmid pSD19, the deletion derivative of pSAlB-56A that contains the smallest insert (280 bp) still capable of maximum resolution, to construct unidirectional deletions from the undeleted end of the inverted repeat (Fig. 1).

The 10 "reverse" deletion clones used in further analysis were chosen on the basis of providing an evenly spaced spectrum of deletion endpoints (Fig. 2 and 3). Each of the reverse deletion isolates was tested for the ability to generate linear minichromosomes on transfection into SFV-infected rabbit SIRC cells. The relative efficiencies of resolution of these reverse deletions (Fig. 2 and 3) allowed us to draw several conclusions. First, deletion of DNA sequences up to region III did not affect the efficiency of resolution, but the deletion of even 7 bp of the right-side region III caused a dramatic drop-off in resolution efficiency. Second, the deletion of most of the short palindrome between regions I and III (see Fig. 3A and C) reduced resolution to barely detectable levels. Third, the deletion of region I completely abolished resolution. Fourth, with one exception the results of the reverse deletions shown in Fig. 2 and 3 are qualitatively identical to the data obtained with deletions from the other end of the inverted repeat (see Fig. 3B and C). The one major difference between the two sets of deletions was the effect of the presence of region II (Fig. 3) on the efficiency of resolution. Whereas the inclusion of this region at the left end of the inverted repeat markedly increased the efficiency of resolution (Fig. 3B; 12), a corresponding increase was not observed with the reverse deletions at the right side of the axis of symmetry (Fig. 3C). Finally, deletion of part of the right region III resulted in a much-reduced efficiency of resolution in the pSD19 reverse deletions but only slightly affected resolution of similar deletions of pSAlB-56A at the left side of the axis of symmetry.

Bidirectional central-axis deletions. The central 64 bp of the inverted repeat arrangement of the SFV hairpin terminus contains 8 nonpalindromic bp (12). To determine whether these AT-rich central sequences are required for telomere resolution, we constructed a set of bidirectional deletions starting from the axis of symmetry. The construction of these central-axis deletions was facilitated by the presence of a fortuitous unique *Afl*II restriction enzyme recognition site (CTTAAG) at the axis of symmetry. Since a proportion of the extracted plasmid clones containing the SFV telomeric palindrome were found to be extruded in the cruciform configuration (12; P. Dickie, A. R. Morgan, and G. McFadden, J. Mol. Biol., in press) and were thus resistant to digestion by *Afl*II, pSD19 was first partially relaxed to the lineform with topoisomerase I before the *Afl*II digestion. The *Afl*II-linearized pSD19 was separated from the remaining circular molecules (that had not resorbed into the lineform conformation) by preparative gel electrophoresis in the presence of ethidium bromide. Bidirectional deletions were then generated from the *Afl*II 5'-TTAA overhang with exonucleases III and VII as described in Materials and Methods.

Five central-axis deletion plasmids (pD5-16 [Δ27], pD6-3 [Δ71], pD5-10 [Δ108], pD5-1 [Δ180], and pD6-20 [Δ169]; see Fig. 6B for schematic comparison of their boundaries) were separately transfected into SFV-infected cells, and the proportion of linear minichromosomes was determined in each case (Fig. 4). Both pD5-16 and pD6-3 were as efficient as the parental pSD19 in their ability to induce resolution of the inverted repeat to daughter hairpin structures. On the other hand, pD5-10, which has 37 more nucleotides deleted than pD6-3, resolved very inefficiently, and pD5-1 and pD6-20 did not resolve at all.

Central-axis additions. The observation that deletion pD6-3, which has most of the central AT-rich nonpalindromic region deleted, still resolved efficiently suggests that these central sequences are not required for efficient resolution. With the aim of reconstructing the minimal DNA sequence required for efficient resolution, we used the central-axis deletion plasmid pD5-10, which is resolved to linear minichromosomes with a very low efficiency, as a substrate for insertional mutagenesis. pD5-10 is particularly well suited for this purpose because it contains a perfect palindrome with a unique *Hpa*I site (GTTAAC) at its axis of symmetry. This plasmid, which was generated by the fortuitous precise removal of 57 nucleotides from the left axis and

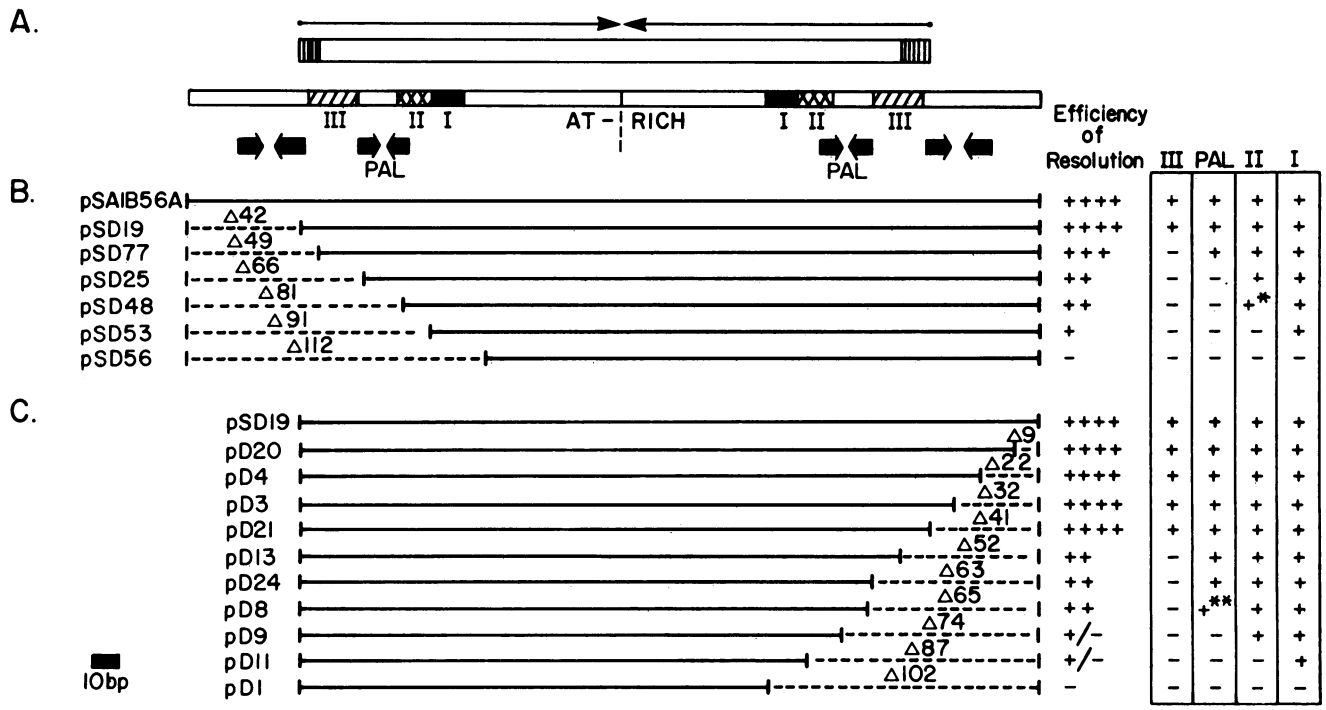

FIG. 3. Summary of reverse unidirectional deletions from both sides of the axis of symmetry of the SFV telomere. The 322-bp SFV telomeric inverted-repeat insert of pSAIB-56A is schematically shown in panel A; I, II, III, and PAL refer to conserved sequence regions described by DeLange et al. (12). The AT-rich central region between the left and right copies of region I contains 8 nonpalindromic bp and therefore displays imperfect symmetry. The unidirectional deletions of pSAIB-56A in panel B have been described previously (12) and are illustrated here for comparison. The unidirectional deletions of pSD19, itself a 42-bp deletion derivative of pSAIB-56A, are illustrated in panel C. The efficiencies of resolution of each of these deletions, when transfected in SFV-infected SIRC cells, are indicated at the right and are derived from relative estimates of the frequency of resolved minichromosomes on Southern blots (12; Fig. 2). The panel at the far right indicates whether a particular conserved DNA region has been partially or completely deleted (−) or retained (+). The diagram at the top summarizes the results of experiments illustrated in parts B and C. The box defines the outer boundaries of the DNA segment required for efficient resolution in SFV-infected cells. The actual boundaries lie within the region indicated by vertical bars. Symbols: *, 2 bp of region II have been deleted; **, 1 bp of PAL has been deleted.

51 nucleotides from the right axis of the native telomeric inverted repeat, includes region I plus 4 additional bp at each side of the axis of symmetry (see Fig. 6 and 7). Insertion of synthetic oligonucleotides in the *Hpa*I site of pD5-10 was achieved by relaxation of the plasmid with topoisomerase I as previously described with pSD19, cleavage with *Hpa*I, and the isolation of linearized pD5-10 molecules by preparative gel electrophoresis in the presence of ethidium bromide. Since *Hpa*I produces blunt ends, this pD5-10 linear could be directly blunt-end ligated with a variety of defined double-stranded oligonucleotides.

For the first experiment, we selected a palindromic 22-mer which, when inserted in the *Hpa*I site of pD5-10, correctly replaces 11 nucleotides of the original telomeric inverted repeat at both sides of the central axis and also reconstitutes a unique *Afl*II site at its new axis of symmetry. The construct pSAD-2, containing this synthetic oligonucleotide at its axis, was then transfected into SFV-infected cells and shown to resolve to minichromosomes as efficiently as pSD19 and the smaller deletions pD5-16 and pD6-3 (Fig. 5, lanes 1 to 5). This observation provides firm evidence that neither the central AT-rich region nor the presence of nonpalindromic base pairs is required for the efficient resolution to hairpin termini. To rule out the possibility that the synthetic 22-mer insert acted as a nonspecific spacer, we inserted several synthetic linkers in the *Hpa*I site of pD5-10. Insertion of one, two, three, and five copies of the *Bam*HI linker octomer 5′-CGGATCCG-3′ or of a single copy of the *Eco*RI-*Hha*I adaptor octomer 5′-AATTCGCG-3′ (Fig. 5, lanes 6 to 10) did not result in obvious enhancement of resolution. Finally, two unrelated plasmids containing palindromic inserts which readily form cruciform structures in vitro (see reference 14 for discussion of facile cruciform transitions) were also transfected into SFV-infected cells. Both pAT34 (Fig. 5, lane 11), which contains a 34-mer stretch of alternating A-T (15), and pAL114 (not shown), which contains a perfect 114-nucleotide palindrome (25), failed to generate detectable levels of resolution products.

DISCUSSION

The hairpin terminus of the genome of SFV has been cloned in circular vectors in recombination-deficient *E. coli* as a 322-bp imperfect inverted repeat (12). When a plasmid containing this inverted repeat was transfected into SFV-infected cells, it served as a substrate for *trans*-acting virus-induced proteins which efficiently converted the circular plasmid into a linear minichromosome with bona fide viral hairpin termini (12). We used unidirectional deletions at both ends of the inverted repeat to show that the *cis*-acting DNA sequence required for this resolution event included about 120 bp at each side of the axis of symmetry (Fig. 3). By making use of bidirectional deletions and insertions of synthetic oligonucleotides at the axis of symmetry, we have, in

addition, demonstrated that at least 86 bp of the central AT-rich region, which includes all 8 nonpalindromic bp, can be deleted without affecting the efficiency of resolution of the inverted repeat. These studies define the *cis*-acting sequence as two identical inverted copies of a DNA region that is between 58 and 76 bp long (Fig. 6 and 7).

The *cis*-acting DNA element required for the efficient resolution of the inverted-repeat conformation displays several sequence features that closely resemble the analogous region of another poxvirus, vaccinia virus. The conserved regions I (11 bp), II (13 bp), PAL (19 bp), and III (18 bp) have been previously described (12). In addition to these elements of sequence conservation, we detected the same 5 bp 5′-TAAAT-3′ 4 bp distal to region I of vaccinia virus and 5 bp distal to the same region of SFV. Because at least part of this sequence is required for efficient resolution, it is plausible that its conservation is another significant feature of poxviral telomere resolution, and we have designated it region IA (Fig. 7).

The relative contributions of regions IA, I, II, PAL, and III have been assessed by using unidirectional deletions at each end of the inverted repeat. The two copies of region I and an adjacent sequence, which may include the conserved region IA, in the intervening AT-rich region appear to constitute a minimal core region required for a low basal level of resolution. The adjacent regions II, PAL, and III are required to obtain the highly efficient resolution that is observed in vivo. We observed two apparent discrepancies between the resolution of unidirectional deletions determined in this study and of those at the opposite side of the inverted repeat, reported earlier (12). First, deletion of part or all of region II at one side of the axis of symmetry in plasmid pSAlB-56A, but not at the other side of the axis in pSD19, results in a marked drop in the efficiency of resolution (Fig. 3). Second, deletion of region III or a part thereof at one side of the axis of symmetry in pSAlB-56A results in a slight reduction in the efficiency of resolution, whereas a similar deletion at the opposite side of the inverted repeat in pSD19 results in a substantially reduced efficiency of resolution (Fig. 3). These apparent discrepancies could be caused by adjacent vector sequences in the constructs used. It is, however, also plausible that these differences are caused either by asymmetry due to nonpalindromic base pairs near the axis of symmetry or by the presence of a 42-bp region at one end of the inverted repeat, distal to region III, in pSAlB-56A, which has been deleted in pSD19. Our data clearly demonstrate that neither the region distal to region III on either side of the symmetry axis nor the central AT-rich region containing the nonpalindromic base pairs is required for efficient resolution. Whatever role these regions may have in resolution, it is obviated by the presence of the 58- to 76-bp core target element at each side of the axis of symmetry (Fig. 6 and 7), at least in the test system used here.

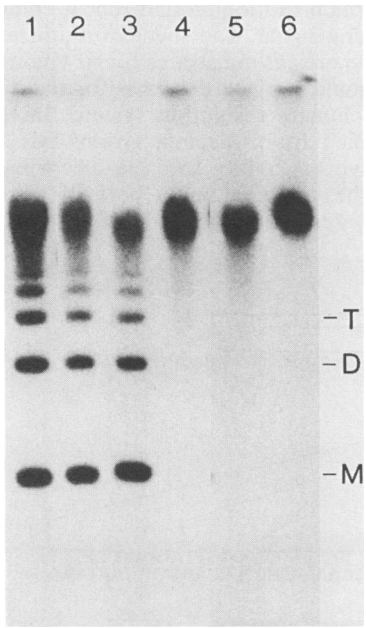

FIG. 4. Resolution of central-axis deletions of pSD19. Plasmid pSD19 and five central-axis deletion derivatives (diagrammed in Fig. 6B) were transfected into SFV-infected cells, and the DNA was extracted after 24 h, electrophoresed in 0.7% agarose gel, blotted onto nitrocellulose paper, and hybridized with pUC DNA as described in the legend to Fig. 2. Lanes 1 to 6: plasmid pSD19 and deletions pD5-16, pD6-3, pD5-10, pD5-1, and pD6-20, respectively. The resolved linear minichromosomes are monomers (M), dimers (D), trimers (T), and higher-order oligomers. An overexposure of this blot (not shown) indicated a low level of resolved minichromosomes for pD5-10 but none for pD5-1 and pD6-20.

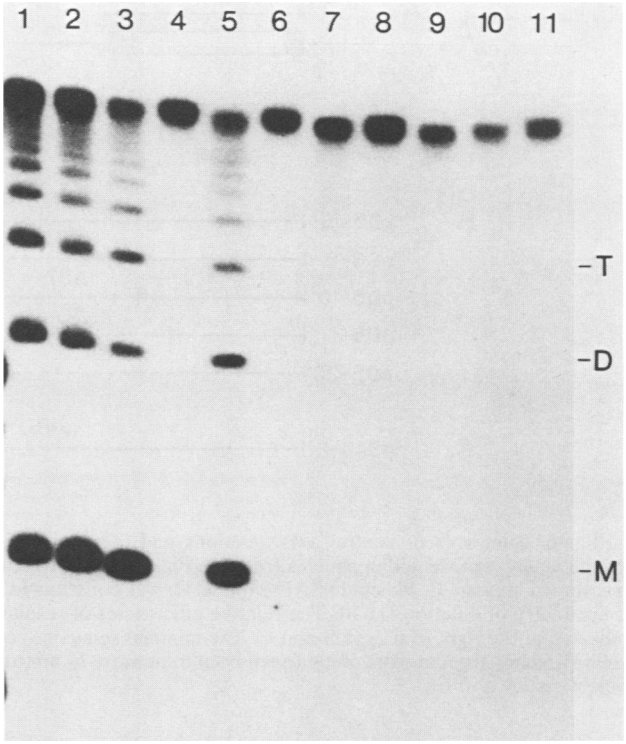

FIG. 5. Resolution of central-axis additions of the pD5-10 deletion derivative. Plasmids were transfected into SFV-infected cells, the DNA was extracted after 24 h, and 250-ng samples were electrophoresed, blotted, and hybridized with a vector probe as described in the legend to Fig. 2. Lanes: 1, pSD19; 2, pD5-16; 3, pD6-3; 4, pD5-10; 5, pSAD-2; 6, pSA5/10-A27; 7, pSA5/10-B32; 8, pSA5/10-B17; 9, pSA5/10-B24; 10, pSA5/10-B27; and 11, pAT34. Monomer (M), dimer (D), and trimer (T) minichromosomes are indicated. Lanes 5 to 10 contain, respectively, the following inserts at the central *Hpa*I site of pD5-10: palindromic 22-mer (ATAAA TTACTTAAGTAATTTAT); one copy of *Eco*RI-*Hha*I adaptor 8-mer; one copy of *Bam*HI linker 8-mer; two copies of *Bam*HI linker; three copies of *Bam*HI linker; and five copies of *Bam*HI linker.

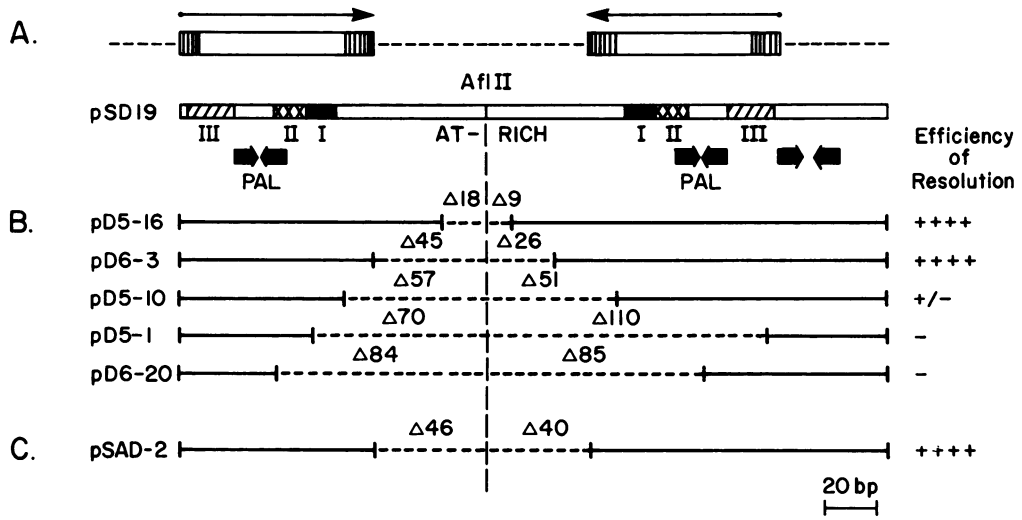

FIG. 6. Summary of central axis deletions and insertions of the SFV telomeric sequences. The inverted-repeat insert in pSD19 is schematically represented in panel A (see also Fig. 3). The *Afl*II site at the axis of symmetry was used to construct the deletions at the central axis shown in panel B. Plasmid pSAD-2 (panel C) was constructed by insertion of a synthetic 22-mer palindrome into the *Hpa*I site at the axis of symmetry of deletion D5-10. The relative efficiencies of resolution of each plasmid were estimated from Southern blots (Fig. 5) and are indicated at the right of panels B and C. The minimal sequence required for maximal resolution to viral hairpin termini, shown at the top of panel A, spans from at least 58 bp (open box) to at most 76 bp (total box), at each side of the axis of symmetry. The DNA sequence of this region is shown in Fig. 7.

The termini of poxvirus genomes can now be subdivided into at least two functional domains, namely, the terminal AT-rich domain which contains the extrahelical bases (1, 12) and the adjacent 58- to 76-bp domain which is required for efficient resolution of the inverted repeat replicative intermediate to viral hairpin termini. This strict conservation both of AT richness and of the characteristic extrahelical bases within the terminal domains of the two poxviruses SFV and vaccinia virus, which are members of different genera, implicates these features of the viral hairpin in some aspect of viral replication. Since the terminal AT-rich domain has been deleted without any effect on the resolution of the inverted repeat to the hairpin conformation, we postulate that this domain performs some other crucial function. Candidates for such a function are (i) attachment of the viral genome to a hypothetical virion or virosome matrix; (ii) an origin of replication of the viral genome; (iii) the conversion of hairpin termini to the inverted repeat conformation; and (iv) a packaging signal. The successful utilization of cloned poxvirus telomeres as substrates for in vivo replication and resolution should provide the basis for the development of an in vitro telomere resolution system. In this regard, an enzyme purified from vaccinia virions has recently been shown to have the ability to cross-link substrate DNA in vitro (17). The combination of both in vivo and in vitro replication systems should provide the tools with which to

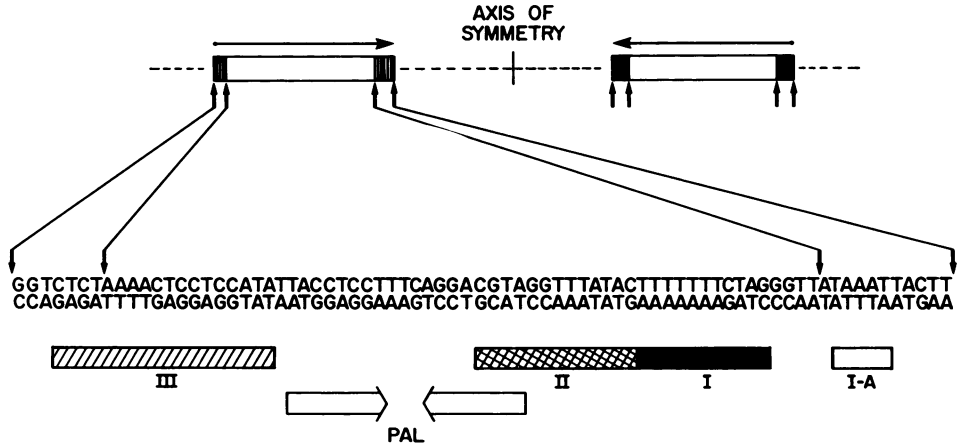

FIG. 7. Minimal DNA target region required for resolution of the SFV hairpin telomere. The two horizontal black arrows indicate the identical inverted DNA segments that are required for resolution of the SFV inverted repeat to the native hairpin conformation. The left and right boundaries of each segment are located between the two sets of vertical arrows. The left boundary was determined by deletion analysis, and the right boundary was determined by insertion of the synthetic 22-mer palindrome 5'-ATAAATTACTT/AAGTAATTTAT-3' in the *Hpa*I site at the axis of symmetry of deletion pD5-10. Regions I, II, PAL, and III have extensive homology with analogous regions required for resolution of the vaccinia virus hairpin terminus and were described by DeLange et al. (12). Region IA is perfectly conserved in the corresponding region of vaccinia virus but at 4 instead of 5 bp from region I.

decipher the functions and mechanisms used by the poxvirus termini during viral DNA replication.

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