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Shope Fibroma Virus DNA Topoisomerase Catalyses Holliday Junction Resolution and Hairpin Formation *in Vitro*

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The telomeres of poxviral chromosomes comprise covalently closed hairpin structures bearing mismatched bases. These hairpins are formed as concatemeric replication intermediates and are processed into mature, unit-length genomes. The structural transitions and enzymes involved in telomere resolution are poorly understood. Here we show that the type I topoisomerase of Shope fibroma virus (SFV) can promote a recombination reaction which converts cloned SFV replication intermediates into hairpin-ended molecules resembling mature poxviral telomeres. Recombinant SFV topoisomerase linearised a palindromic plasmid bearing 1.5 kb of DNA encoding the SFV concatemer junction, at a site near the centre of inverted-repeat symmetry. Most of these linear reaction products bore hairpin tips as judged by denaturing gel electrophoresis. The resolution reaction required palindromic SFV DNA sequences and was inhibited by compounds which block branch migration ($MgCl_2$) or poxviral topoisomerases. The resolution reaction was also slow, needed substantial quantities of topoisomerase, and required that the palindrome be extruded in a cruciform configuration. DNA cleavage experiments identified a pair of suitably oriented topoisomerase recognition sites, 90 bases from the centre of the cloned SFV terminal inverted repeat, which may mark the resolution site. These data suggest a resolution scheme in which branch migration of a Holliday junction through a site occupied by covalently bound topoisomerase molecules, could lead to telomere resolution.

Keywords: poxviral topoisomerase; telomere resolution; Holliday junction; hairpin formation; site-specific recombination

Introduction

Poxviral chromosomes consist of linear DNA duplexes bounded by hairpin termini (Baroudy *et al.*, 1982) These hairpin structures are formed late in infection as concatemeric viral replication products are cut into mature viral chromosomes, condensed with protein, and packaged (DeLange & McFadden, 1990; DeLange *et al.*, 1986; Merchlinsky & Moss, 1986). Although the mechanics of telomere resolution are broadly understood, the enzymes which catalyse resolution remain to be identified. The DNA structures targeted by these enzymes are also unknown.

Abbreviations used: SFV, Shope fibroma virus; TIR, telomeric inverted repeat.

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Transfection studies have shown that the resolution reaction is dependent upon specific DNA sequences (DeLange & McFadden, 1987; Merchlinsky, 1990b; Merchlinsky & Moss, 1989b). These sequences are conserved among a variety of poxviral species and are located near the centre of inverted-repeat symmetry which forms the concatemer junction between newly replicated poxviral chromosomes. The most highly conserved of the sequences essential for resolution has been termed the ''res-site'' (Merchlinsky, 1990b) and was shown to be a functional late promoter oriented in such a way as to permit transcription across the concatemer junction (Hu & Pickup, 1991; Stuart *et al.*, 1991). Deletion analyses have identified other conserved sequence elements which play a lesser role in the resolution reaction and serve an unknown function.

These transfection studies have also shown that inverted-repeat symmetry is an additional, and

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essential, feature of the resolution substrate (DeLange & McFadden, 1987). This is compatible with the proposal that poxviruses might generate mature hairpin telomeres by a process which involves a cruciform extrusion reaction followed by cleavage of the resulting Holliday junction (McFadden & Morgan, 1982; Merchlinsky, 1990a). Such a scheme is energetically practical (Dickie *et al*., 1987, 1988), provides a simple way of ensuring symmetrical cleavage, and can explain the origin of the mismatched bases which are found immediately adjacent to mature hairpin termini. This is because the inverted repeats generated by DNA replication are not perfectly symmetrical. Consequently, the strand-transfer reactions associated with cruciform extrusion are expected to generate such mismatches wherever imperfectly symmetrical bases are juxtaposed by hairpin extrusion.

A number of studies have attempted to identify the enzymes that catalyse poxviral telomere resolution reactions. Genetic screens have shown that the resolution reaction requires a late gene product and/or the late transcription machinery itself (Carpenter & DeLange, 1991; DeLange, 1989; Merchlinsky, 1989; Merchlinsky & Moss, 1989a). Nucleases have also been purified from viral particles and infected cells which can resolve plasmids into linear molecules with cross-linked or hairpin ends (Lakritz *et al*., 1985; Merchlinsky *et al*., 1988; Stuart *et al*., 1992). However, none of these nucleases show the sequence specificity which seems to be an essential feature of the resolution reaction. The genetic origin of these enzymes is also obscure.

Although endonucleolytic attack has been favoured as a method of poxviral telomere resolution, other mechanisms can be envisioned. Merchlinsky *et al*. (1988) first suggested that topoisomerase-like breakage and reunion reactions might play some role in telomere dynamics, and subsequent research into the transesterification reactions catalysed by vaccinia (Sekiguchi *et al*., 1996; Shuman, 1989, 1991b) and other (Klemperer *et al*., 1995; Palaniyar *et al*., 1996) poxviral topoisomerases has provided data compatible with this hypothesis. Here, we show that Shope Fibroma virus (SFV) topoisomerase can catalyse a hairpin-forming reaction using substrates bearing the replicative form of the SFV telomeric inverted repeat (TIR). To our knowledge this is the first demonstration that poxviral hairpins can be generated by an enzyme of known poxviral origin. Nevertheless, a topoisomerase-dependent resolution scheme still cannot fully account for the known properties of poxviral telomeres.

Results

Resolution of plasmids encoding the SFV TIR

Plasmid pSCB-1a bears a 1.5 kb insert in pUC19 encoding the replicative form of the SFV telomere (Figure 1). The insert has been shown to be a telomere resolution substrate in transfection studies

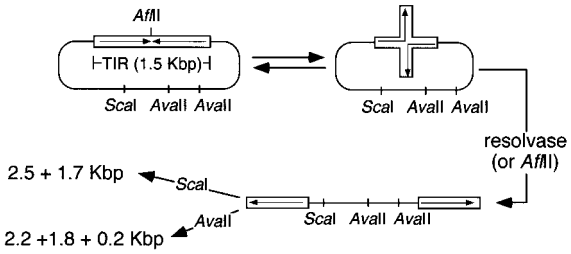

Figure 1. Assaying telomere resolution using plasmid pSCB-1a. In a typical resolution assay the DNA is treated with topoisomerase and then digested with *Sca*I. This produces 2.5 and 1.7 kb restriction fragments which are diagnostic for the resolution reaction. In one experiment (Figure 6) the topoisomerase-treated DNA was digested with *Ava*II, in which case 2.2, 1.8, and 0.22 kb reaction products were expected. Marker DNAs were prepared by substituting *Afl*II for the topoisomerase.

(DeLange *et al*., 1986) and interconverts between lineform and cruciform structures *in vitro* (Dickie *et al*., 1987, 1988). To test whether this plasmid was a resolution substrate *in vitro*, we applied purified SFV topoisomerase to a heparin affinity column, eluted the protein with a salt gradient, and then incubated the topoisomerase-containing fractions with pSCB-1a. The reaction products were digested with *Sca*I to map the cleavage point, and then subjected to agarose gel electrophoresis. We observed a peak of resolution activity that co-eluted with both topoisomerase protein and topoisomerase activity from this column (Figure 2). The fragment sizes (1.7 and 2.5 kb) were consistent with an endpoint located very near the centre of palindromic symmetry.

Reaction requirements and the effects of inhibitors

To better characterise the reaction requirements, SFV topoisomerase was incubated with other DNA substrates under various reaction conditions (Figure 3). We noted that resolution activity was undetectable in cell extracts isolated from vector-transformed *E. coli* cells (lane 2) and required only a Tris-HCl buffer in standard reactions (lane 3). The reaction was strongly inhibited by 3 mM $MgCl_2$ (lanes 4 and 5), but not by 100 mM NaCl (lanes 6 and 7), implying that inhibition is magnesium-specific. Because the degree of plasmid extrusion varies somewhat from stock to stock and might have some effect on reaction efficiency, we heated pSCB-1a for one hour at 55 °C in the presence of 100 mM NaCl, cooled the reaction, and then added topoisomerase. This treatment decreased the proportion of cruciform plasmids by about half (data not shown), but still had little effect on resolution efficiency (compare lanes 7 and 9). *Sca*I-linearised pSCB-1a DNA was not cut by the enzyme (lanes 10 and 11), nor were plasmids

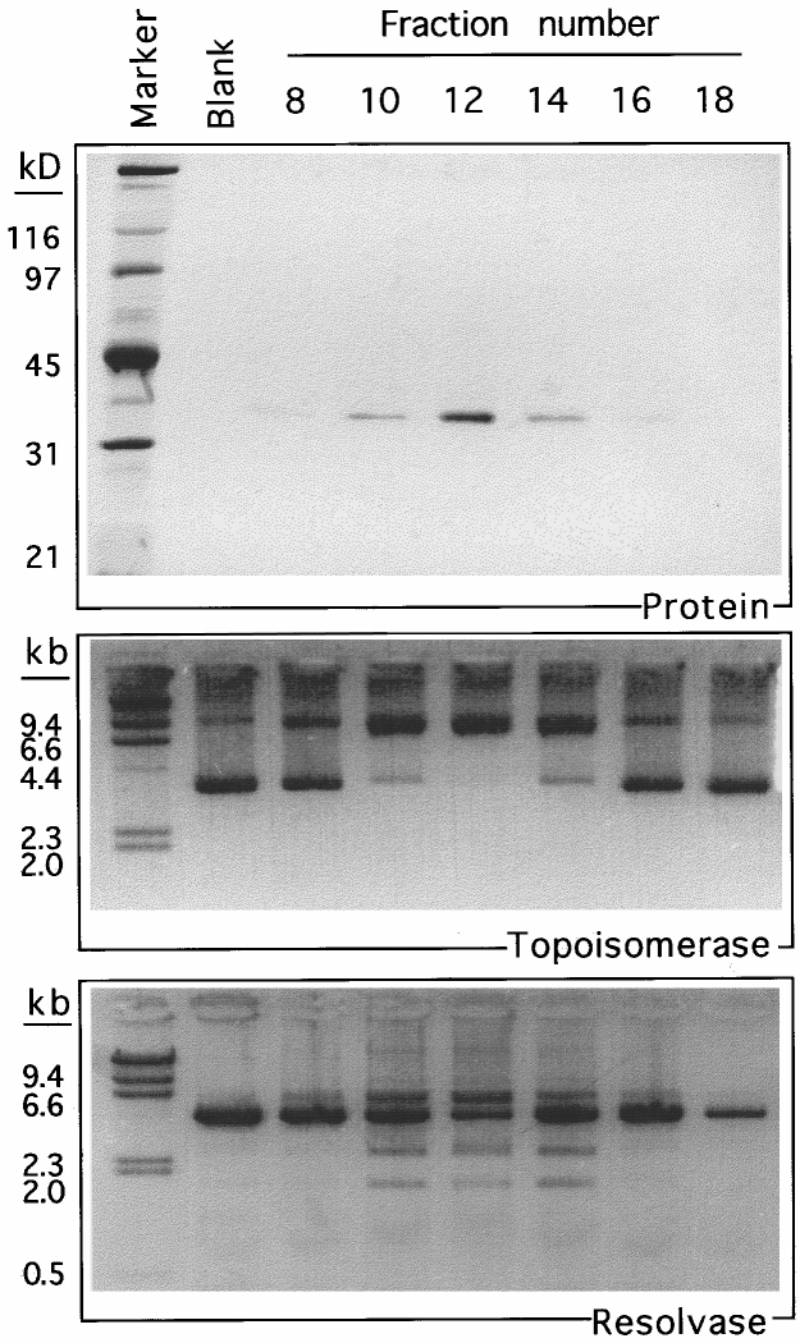

Figure 2. Co-elution of SFV topoisomerase with a telomere resolution activity. SFV topoisomerase (fraction IV) was applied to a 5 ml HiTrap heparin column, washed, and eluted with a NaCl gradient (30 ml, 0.05 M-1.5 M). Topoisomerase-containing fractions were subjected to SDS-PAGE analysis (10 µl per lane) and the protein detected using a silver stain (upper panel). These same fractions were also assayed for the capacity to relax pBluescript DNA (0.1 µl per reaction, middle panel), or resolve pSCB-1a DNA (5 µl per 20 µl reaction, lower panel).

bearing a small inverted repeat of non-poxviral origin (lanes 12 and 13), or the pUC19 vector from which pSCB-1a derives, cut by SFV topoisomerase.

Several drugs have been shown to inhibit poxviral topoisomerase activity at micromolar concentrations. We noted that these compounds also inhibited resolution at concentrations comparable to those which inhibit topoisomerization. Ethidium bromide was the most potent inhibitor of resolution, causing half-maximal inhibition at 1-2 µM concentration in the presence of 45 µM pSCB-1a nucleotide (Figure 4). Berenil (diminazene aceturate) and actinomycin D were also inhibitory at concentrations of ~15 and ~70 µM, respectively. These concentrations of ethidium bromide and actinomycin D would be expected to unwind 10-20 and 160-170 turns of DNA helix, respectively. Consequently, the two intercalating agents showed no obvious correlation between unwinding activity and inhibitory activity. We also noted that subinhibitory concentrations of these drugs consistently enhanced plasmid resolution activity up to 1.4-fold (Figure 4).

Kinetics and stoichiometry

The reaction showed time-dependent resolution kinetics, but occurred slowly *in vitro* and required substantial quantities of topoisomerase. Figure 5 shows the effect of varying the topoisomerase con-

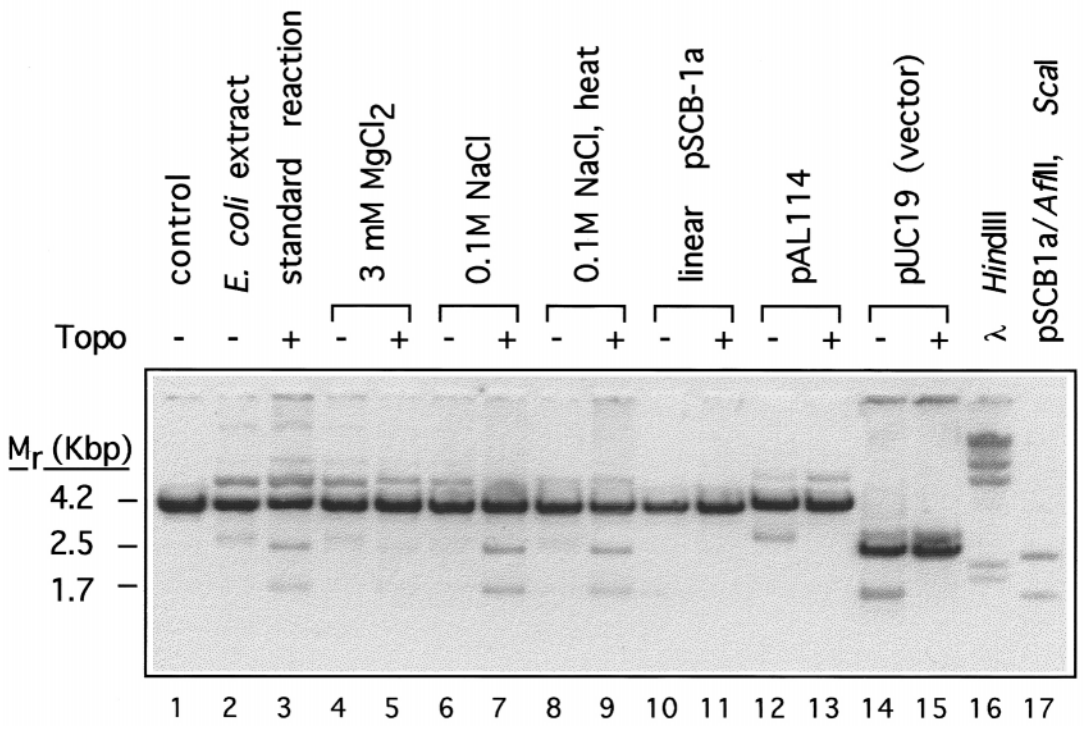

Figure 3. Resolution reaction requirements. Resolution reactions contained 2 μg of DNA, SFV topoisomerase (0 or 5 μg), and the indicated supplements in a total volume of 80 μl. The reactions were incubated at 37 °C for one hour, stopped, phenol extracted, precipitated, and the DNA digested with *Sca*I prior to agarose gel electrophoresis. Lane 1, pSCB-1a substrate, no treatment; lane 2, 1 μl (0.6 ng) *E. coli* vector control protein; lane 3, standard reaction; lanes 4 and 5, 3 mM added $MgCl_2$; lanes 6 and 7, reactions supplemented with 0.1 M NaCl and held on ice for one hour prior to topoisomerase addition; lanes 8 and 9, reactions supplemented with 0.1 M NaCl and incubated one hour at 55 °C prior to cooling and topoisomerase addition; lanes 10 and 11, *Sca*I-linearised pSCB-1a substrate; lanes 12 and 13, pAL114 substrate; lanes 14 and 15, pUC19 substrate; lanes 16 and 17, DNA size markers. The faint bands migrating at ~2.6 kb (lanes 2, 4, 6, 8, and 12) are incompletely restricted form I molecules which disappear upon relaxation by topoisomerase (see Materials and Methods).

centration while the amount of pSCB-1a substrate remained fixed at 100 μg/ml. Maximal cleavage was obtained using a ~160:1 molar ratio of protein:plasmid (Figure 5, left panel). This corresponded to a protein:target molar ratio of less than eight because the substrate encodes 21 copies of the 5′ [C/T]CCTT 3′ motif, plus an unknown number of motif variants that may be recognisable by poxviral topoisomerases (Shuman, 1991a; Shuman & Prescott, 1990). Under these same optimal conditions, the reaction displayed non-linear resolution kinetics. A slight lag was noted during the first few minutes of the reaction, after which a maximal resolution rate ~0.4 pmol/hour was transiently observed (Figure 5, right panel). Up to 40 % of the substrate was cut into linear resolution products over a three hour time-course.

Resolution products contain hairpin structures

If the reaction catalysed by SFV topoisomerase *in vitro* reproduces that which generates mature poxviral telomeres *in vivo*, one would expect the resolution products to bear hairpin tips. To examine this feature of the reaction, we 3′ end-labeled and gel-purified the 1.8 and 2.2 kb DNA fragments produced by combined treatment with topoisomerase and *Ava*II (see Figure 1; *Sca*I leaves blunt ends which are difficult to label). These DNA fragments were then size-fractionated by electrophoresis through alkaline agarose gels (Figure 6). Marker DNA fragments were produced by digesting pSCB-1a with *Afl*II and *Ava*II (Figure 1), and labeling the product in an identical manner. It was observed that whereas nearly all the DNA digested with *Afl*II and *Ava*II ran as single-stranded molecules of 1.8 or 2.2 kb in length, most of the labeled DNA treated with topoisomerase and *Ava*II ran as dimeric or ''cross-linked'' species (Figure 6). Thus, most of the resolution products bear hairpin tips resembling poxviral telomeres.

DNA sequence and topological requirements

Transfection studies have shown that SFV telomere resolution reactions are dependent upon sequences encoded within ~100 bp of the concatemer junction (DeLange *et al.*, 1986). To determine which of these sequences might be necessary for resolution *in vitro*, we obtained plasmids encoding different portions of the SFV and vaccinia virus termini (Figure 7) and assayed for topoisomerase-mediated resolution under otherwise standard reaction conditions. These experiments showed

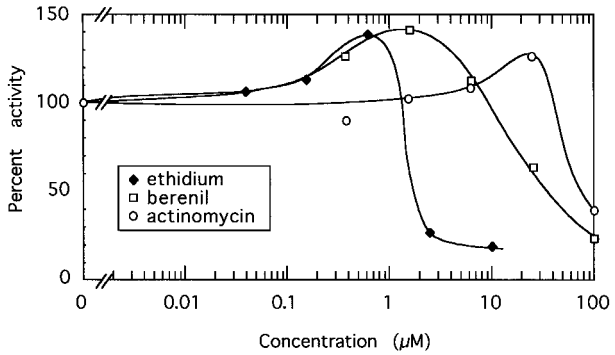

Figure 4. Effects of inhibitory drugs. Resolution reactions contained 1 μg of fraction IV topoisomerase, 0.6 μg pSCB-1a, and the indicated concentrations of topoisomerase-inhibiting drugs in 50 μl. After two hours at 37 °C, the DNAs were extracted with phenol and precipitated, digested with *Sca*I, fractionated by agarose gel electrophoresis, and the DNA distribution determined by densitometry. One hundred per cent activity in this experiment corresponded to about 25 % resolution.

that pSCB-1a, which contains 1.5 kb of inverted repeat, was the only functional resolution substrate. A plasmid encoding half of the SFV TIR sequences needed for resolution *in vivo* was unreactive, although it retained one of the two central topoisomerase recognition sites (pDE-5). So, too, was a plasmid containing an 86 bp symmetric deletion of the bases and topoisomerase recognition sites found at the centre of the SFV TIR (pSAD-2). Plasmids encoding 242 bp of vaccinia virus TIR (VCB-5a), or ~410 bp of the vaccinia virus TIR substituted with one (pEC/55) or two (p55/55) SFV resolution sequences also failed to be resolved (data not shown).

To test whether superhelicity served any purpose in the resolution reaction we restricted pSCB-1a with *Hin*dIII and then recircularised the DNA with T4 ligase. This was done because, as others have also noted (Dickie *et al*., 1987), topoisomerase-mediated relaxation of these plasmids does not eliminate the cruciform from a substantial portion of molecules. The reaction products were gel purified and found to be fully *Afl*II sensitive, thus demonstrating that all the superhelical turns had been eliminated and the SFV inverted repeat had assumed the lineform configuration essential for cleavage at the *Afl*II site (data not shown). However, these covalently closed lineform plasmids were not substrates for the resolution reaction even

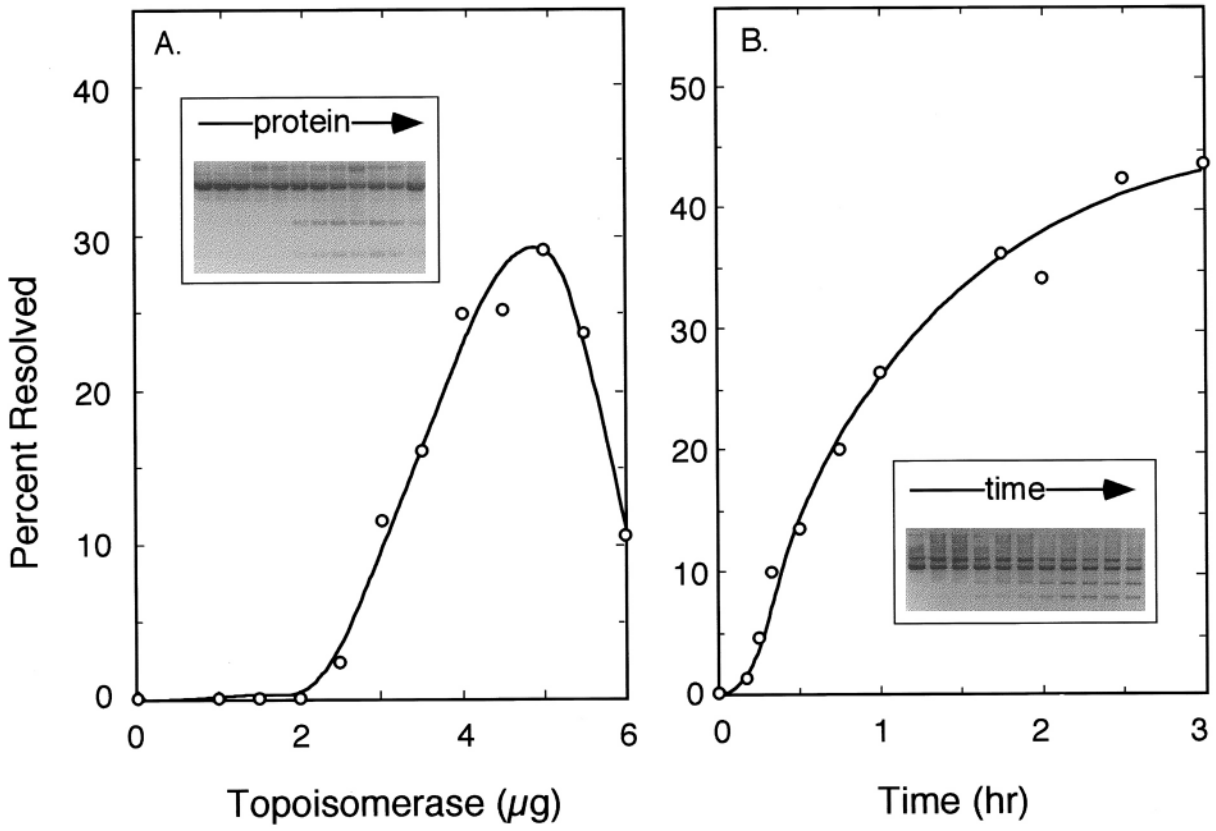

Figure 5. Reaction properties. (a) Stoichiometry. Standard resolution reactions (20 μl) were supplemented with the indicated quantities of fraction V topoisomerase and incubated for two hours at 37 °C. After *Sca*I treatment, the reaction products were separated by agarose gel electrophoresis, stained, photographed, and the DNA distribution determined by densitometry. The scanned gel is shown (inset). (b) Kinetics. Standard reactions (containing 5 μg of fraction V topoisomerase per 20 μl volume) were incubated at 37 °C, stopped at the indicated times, and the percentage resolution determined as described above. The scanned gel is again shown (inset).

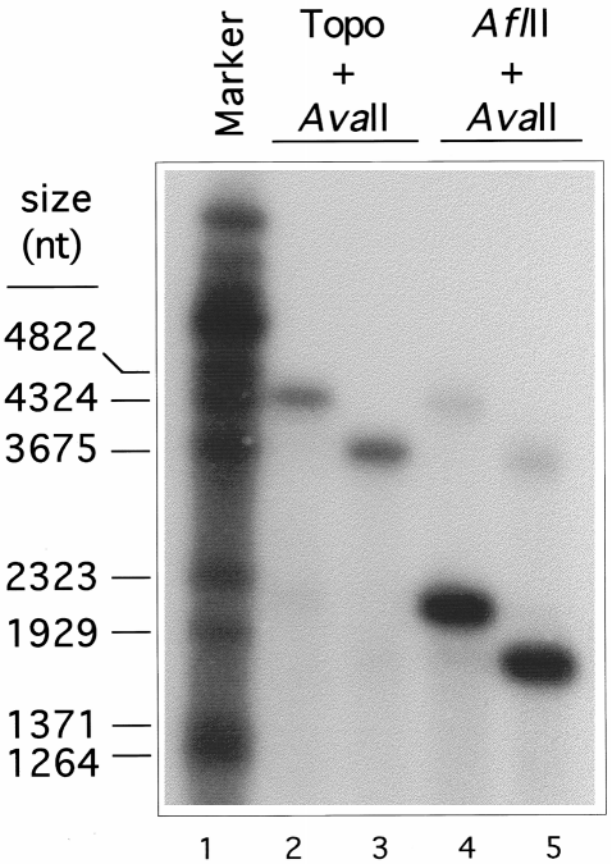

Figure 6. Resolution products bear hairpin tips. pSCB-1a was digested with a combination of either SFV topoisomerase plus *Ava*II, or *Afl*II plus *Ava*II. The reaction products were 3′ end-labeled and the 2.2 and 1.8 kb TIR-containing DNA fragments individually gel-purified using a native agarose gel. The DNA fragments were then heat denatured in the presence of formamide, fractionated on a 0.9 % alkaline agarose gel, fixed, and an autoradiograph prepared. Essentially all of the double-stranded DNAs which were recovered from topoisomerase-containing reactions migrated as either 4400 or 3600 nt single-stranded molecules (lane 2 and 3), whereas DNAs produced by digestion with *Ava*II and *Afl*II primarily migrated as half-length (2200 and 1800 nt) species (lanes 4 and 5). (The faint 4400 and 3600 nt bands seen in lanes 4 and 5 were caused by polymerase labeling of spontaneously nicked molecules.) *Bst*EII-cut λ DNA was end-labeled to produce the markers seen in lane 1.

at very high topoisomerase:plasmid ratios (data not shown).

Hairpin binding and cleavage reactions

The data summarised in the previous section suggested that the resolution target might be the two topoisomerase-recognition sites located at the tips of the cruciform configured TIR. This was surprising as at least two ''downstream'' bases are needed to permit efficient cleavage of duplex-ended oligonucleotides by the vaccinia enzyme (Shuman, 1991a). To test whether SFV hairpin tips really are topoisomerase cleavage sites, we cut pSCB-1a with *Xho*I and then 3′ end-labeled the 707 bp TIR-containing fragment. Some of this lineform DNA was then heat denatured and snap cooled to generate hairpin-ended molecules. The two structural isomers were incubated with varying concentrations of SFV topoisomerase, the covalent complexes cleaved by treatment with SDS, and the reaction products sized on sequencing gels. As expected, most of the topoisomerase-cleavage sites were recognised equally well on both substrates (Figure 8), including a ~440 base product produced by cleavage of the two canonical recognition sites located 90 bp to the 5′ side of the TIR centre. Other common cleavage sites mapped close to the position of variant recognition sites. These included ~230 base products produced by cleavage of two overlapping sites located about 130 bp to the 3′ side of the TIR centre (5′ CCTTTCTT 3′) and an unidentified site located very near the 3′-^{32}P label (not seen in Figure 8). However, while the topoisomerase readily cleaved the two central recognition sites in lineform DNA, these sites became immune to cleavage in heat-treated (i.e. hairpin-ended) DNAs (Figure 8, arrow). These data show that the resolution reaction cannot depend upon cleavage of the two recognition sites located at the very tips of the extruded cruciform.

Discussion

We have shown that highly purified SFV topoisomerase catalyses a time-dependent, resolvase-like reaction that generates the hairpin tips characteristic of mature viral chromosomes. Figure 9 illustrates a simple scheme by which this reaction is probably accomplished. This scheme is nearly identical with a telomere resolution model first proposed by McFadden & Morgan (1982), excepting that a topoisomerase, as Merchlinsky and colleagues have speculated (Merchlinsky *et al.*, 1988), promotes strand cleavage and rejoining rather than an endonuclease and DNA ligase. The reaction depends upon the migration of a Holliday junction across two symmetrically located sites, each containing a covalently bound topoisomerase molecule (Figure 9). Strand exchange, rejoining, and topoisomerase loss would generate contiguous, hairpin-ended, molecules (Figure 6) with base mismatches near the hairpin tips. This model, and the data from which it derives, also clearly supports and extends work showing that vaccinia topoisomerase can catalyse the resolution of small, synthetic, Holliday junctions (Sekiguchi *et al.*, 1996). It is also supported by the observation that the catalytic domain of vaccinia virus topoisomerase resemble that of site-specific recombinases like Cre (Cheng *et al.*, 1998).

This reaction scheme is sustained by essentially all of our experimental data. For example, it is

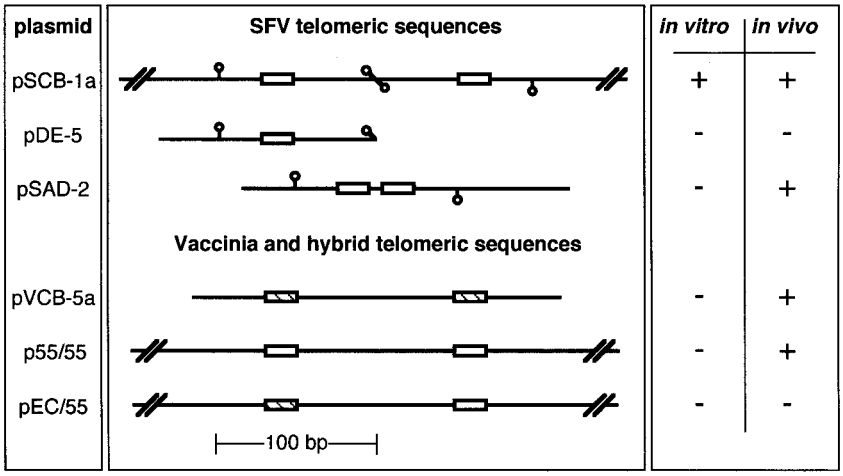

Figure 7. Plasmid substrates. Only the cloned poxviral sequences that are known to play a role in telomere resolution are shown, drawn approximately to scale. Open boxes show SFV res-sites, hatched boxes show vaccinia res-sites, and circles show canonical topoisomerase recognition sites. pDE-5 encodes half of the SFV telomeric inverted repeat including one of the two central topoisomerase recognition sites. pSAD-2 retains the SFV palindrome but incorporates a symmetrical 86 bp deletion of these two topoisomerase targets. pVCB-5a encodes the vaccinia virus TIR, p55/55 and pEC/55 are derivatives of the vaccinia TIR encoding one or two SFV res-sites. The *in vivo* resolution properties of these plasmid substrates are described by DeLange *et al.* (1986) and Merchlinsky & Moss (1989b).

apparent that the cloned TIR must be extruded into a cruciform configuration if it is to serve as a substrate in this reaction. Half of the TIR is not a substrate (pDE-5, Figure 7), and any treatment which permitted the collapse of the TIR back into a lineform configuration (digesting pSCB-1a with *Sca*I alone, or treating the plasmid with *Hin*dIII plus DNA ligase) blocked resolution. A requirement for a branch migrating Holliday junction is also suggested by the fact that $MgCl_2$ strongly inhibits the reaction (Figure 3). $MgCl_2$ has little effect on poxviral topoisomerization reactions, but does dramatically decrease the rate of branch migration (Panyutin & Hsieh, 1994).

For this scheme to work, the base of the pSCB-1a cruciform would also need to be located somewhere near two covalently bound topoisomerase molecules. If all the twist in a 4.2 kb supercoiled plasmid were absorbed into extruded cruciforms (with $\sigma = -0.05$ to -0.07, and ΔLk apportioned 3:7 into ΔTw and ΔWr, respectively; Kornberg & Baker, 1991), one would expect to extrude 30-50 bp of helix into each cruciform arm. Conversely, absorbing all of the superhelical stress into cruciform formation would extrude 100-150 bp arms. The mean lies very close to a canonical cleavage site located 90 bp to the 5′ side of the TIR centre (Figure 8). This is also the location where an array of T7 endonuclease I sensitive-sites, each separated by one turn of helix, have been mapped in a similar plasmid (Dickie *et al.*, 1988). Thus the equilibrium position of the Holliday junction in pSCB-1a would favour resolution by this mechanism.

This *in vitro* reaction is clearly an inefficient one, which requires a high ratio of topoisomerase to substrate. This is illustrated by noting that ~0.5 nmol of SFV topoisomerase was needed to resolve 1 μg of pSCB-1a in 30 minutes at 37 °C (Figure 5) whereas only ~0.06 pmol of topoisomerase would have been needed to remove half of the supercoils from 1 μg of pBR322 in the same time period. Thus resolution is at least 8000-fold slower than is topoisomerisation. Why resolution is so inefficient remains to be determined, although in early studies a high ratio of enzyme-to-substrate was likewise needed when RuvC endonuclease cleaves Holliday junctions in the absence of RuvAB helicase (Bennett *et al.*, 1993). It could be that ordinary cleavage and religation reactions are too rapid to intercept a migrating Holiday junction, or that the plectonemic joints formed at higher protein:DNA ratio (Shuman *et al.*, 1997) are necessary for the efficient telomere resolution. The unusual properties of cruciform-configured SFV TIRs (Dickie *et al.*, 1987, 1988) suggests that there is some structural impediment to unrestricted branch migration within these odd substrates which might also substantially decrease resolution efficiency.

Precisely where resolution occurs *in vitro* is also difficult to establish using these substrates. The two topoisomerase recognition sites located at the very centre of the TIR are not cleaved when incorporated into the hairpin-ended DNAs (Figure 8), which suggests that it is one of the more distal sites which are targeted by the enzyme in cruciform pSCB-1a. An obvious candidate might be the canonical cleavage sites located 90 bp to the 5′ side of the TIR centre (Figure 8). One reason for favouring this location is that it is difficult to imagine how a Holliday junction could migrate through DNA duplexes occupied by one or two topoisomerase molecules. However, this site is positioned in such a way that junctions migrating away from the TIR centre would encounter a correctly oriented topoisomerase-DNA complex. This might also explain why pSAD-2 was not a substrate *in vitro* (Figure 7), because deleting 86 bp surrounding the TIR centre would shift the cross-over point well to

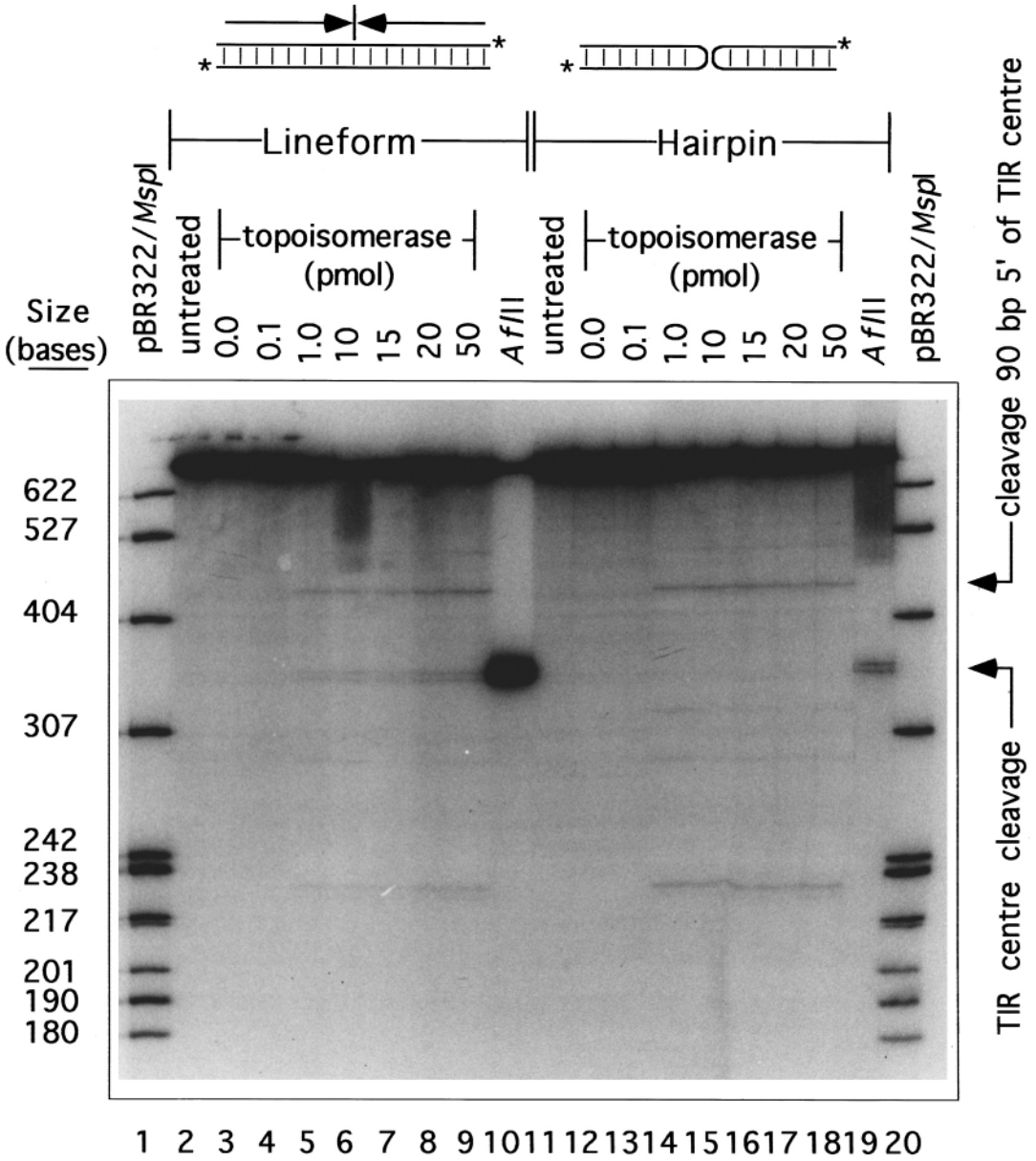

Figure 8. Topoisomerase-mediated cleavage of lineform and hairpin configured DNAs. DNA substrates were 3′ end-labeled and subjected to topoisomerase cleavage in the presence of SDS (Palaniyar *et al*., 1996). The reaction products were size fractionated under denaturing conditions along with ^{32}P-labeled pBR322/*Msp*I size markers. Note the essentially identical cleavage patterns in lanes 3-9 and 12-18, excepting at the two adjacent topoisomerase sites which are found at the very centre of sequence symmetry (arrowed). Cleavage at these two sites is expected to produce molecules nearly identical in size to *Afl*II-cleaved DNA (lane 10). However, these two sites were not cleaved in hairpin-ended molecules (lanes 12-18).

the 5′ side of this site. Experiments like those conducted with *E. coli* RecA and RuvC (Shah *et al*., 1994) would provide better insights into the role of sequence and orientation on topoisomerase-mediated resolution reactions.

SFV topoisomerase promotes a reaction which bears a superficial resemblance to those catalysed by other poxviral ''telomere resolvases''. The first such enzyme to be purified was a virion-borne nuclease, sometimes called ''nicking-joining'' enzyme, which converts pBR322 and cloned vaccinia virus TIRs into linear molecules with hairpin tips (Lakritz *et al*., 1985; Merchlinsky *et al*., 1988; Reddy & Bauer, 1989; Rosemond-Hornbeak *et al*., 1974). However, nicking-joining enzyme is a 50 kDa protein which is too large for it to be vaccinia topoisomerase. Stuart *et al*. (1992) subsequently identified an enzyme in vaccinia-infected cell lysates which also converts plasmids encoding inverted-repeat sequences into linear molecules with hairpin tips. This late protein can be differentiated from nicking-joining enzyme and poxviral topoisomerases, by requiring Mg^{2+} for activity (Bauer *et al*., 1977; Lakritz *et al*., 1985; Shaffer &

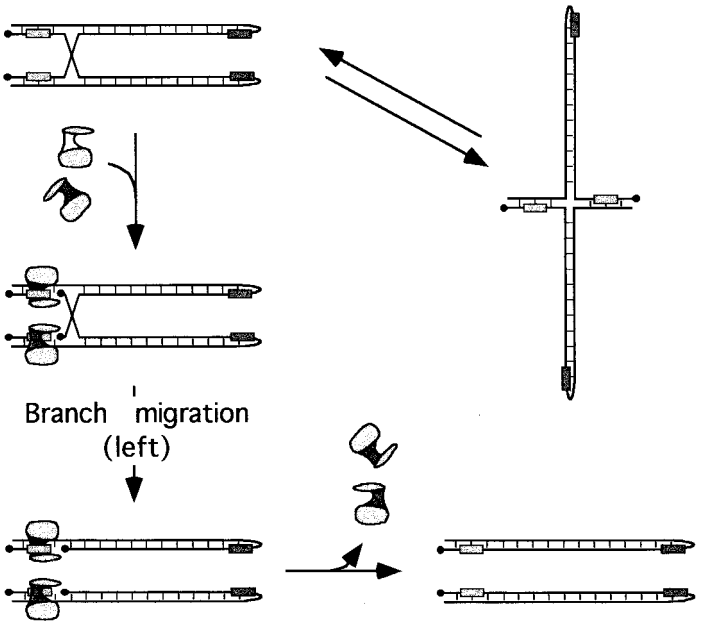

Figure 9. Model for topoisomerase-mediated telomere resolution. Gray boxes indicate canonical topoisomerase recognition sites (5′ (C/T)CCTT 3′) and filled dots indicate the 5′-ended strands. For clarity, the remainder of the plasmid substrate has been omitted. Only the topoisomerases directly involved in resolution are shown.

Traktman, 1987; Shuman & Moss, 1987). The enzyme was also resistant to berenil and catalysed resolution of pSAD-2, a plasmid which is inert in our assays. Although either enzyme could be ''the'' poxviral telomere-resolving activity, or is perhaps part of several redundant resolution pathways operating in infected cells, in neither case do these proteins show the sequence specificity which is a requisite feature of telomere resolution reactions (DeLange *et al.*, 1986; Merchlinsky, 1990a,b; Merchlinsky & Moss, 1989b). Of course these essential sequences may serve another critical function *in vivo*, unrelated to the final resolution step, which is obviated when topologically underwound substrates are employed *in vitro*.

In contrast to these two proteins, the reaction catalysed by SFV topoisomerase is the only one shown to date to require poxviral TIR sequences. Nevertheless, the *in vitro* reaction still does not fully reproduce all of the properties of telomere resolution reactions, as has been established from DNA transfection studies. In particular, neither pSAD-2 nor pVCB-5a are resolved *in vitro* by SFV topoisomerase (Figure 7), although they are substrates when transfected into SFV-infected cells (DeLange *et al.*, 1986). The resolution reaction described here is also appears inefficient compared with the reaction seen in virus-infected cells. These difficulties could simply reflect the fact that *in vitro* reactions rarely reproduce the *in vivo* situation, unless care is taken to reconstitute all of the protein components and to reproduce the properties of the native substrate. One important component, which is missing from our reactions, may be the late transcription machinery (Carpenter & DeLange, 1991; Hu & Pickup, 1991; Merchlinsky & Moss, 1989a; Stuart *et al.*, 1991). Nor can plasmids (or oligonucleotides) perfectly duplicate the structure, mobility, or topology of a newly replicated TIR *in vivo*. Our data support a scheme in which Holliday junctions might migrate away from the centre of a TIR *in vivo*, and be resolved by topoisomerases acting at one of the more distal sites found in all poxviral telomeres. However, proof that topoisomerases play such a role in telomere resolution awaits the construction of topoisomerase-deficient viral mutants and the identification of rate-enhancing accessory factors.

Materials and Methods

Plasmids and other DNAs

Plasmid substrates were obtained from Drs G. McFadden (pDE-5, pVCB-5a, pSCB-1a, and pSAD-2; DeLange *et al.*, 1986; Stuart *et al.*, 1992), M. Merchlinsky (p55/55, and pEC55; Merchlinsky & Moss, 1989b), or laboratory stocks (pAL114; Warren & Green, 1985). Plasmids were purified by alkaline lysis followed by isopycnic centrifugation in CsCl gradients. The ethidium bromide was removed by extraction with butanol and the DNA recovered by precipitation in ethanol or dialysis. Plasmids were stored frozen in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. pSCB-1a stocks prepared in this manner typically contained 20% lineform, 75% cruciform, and <5% nicked species as judged by one and two-dimensional agarose gel electrophoresis.

SFV topoisomerase

A T7 expression plasmid encoding a recombinant form of histidine-tagged SFV topoisomerase (pCF111) was constructed, transformed into *Escherichia coli* strain DE142 [BL21 *recA*; Zhang & Evans, 1995) and protein expression induced as described (Palaniyar *et al.*, 1996). The recombinant protein was purified as follows. A 5.4 l culture was harvested by centrifugation (18,000 *g*, 4 °C) and the cells stored frozen at −80 °C in 35 ml of binding

buffer (0.5 M NaCl, 20 mM Tris-HCl (pH 7.9)) supplemented with protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM benzamidine, 0.1 mM PMSF) and 31 mM imidazole (pH 7.9). The cells were thawed, lysozyme (8 mg) and NP40 (35 μl) were added, and the viscosity reduced by Dounce homogenization and sonication. The solution was clarified by centrifugation (12,000 *g*, ten minutes at 4 °C), the pellet was re-extracted with 60 ml of binding buffer, and then centrifuged again. The two low-speed supernatents were pooled and recentrifuged (380,000 *g*, 30 minutes, 4 °C) producing fraction I (100 ml, 3.5 mg/ml). Fraction I was applied to a 2.5 ml column of His-Bind resin, washed with binding buffer containing 30 mM imidazole and 0.1 mM PMSF, and eluted with binding buffer containing 150 mM imidazole. Topoisomerase-containing fractions were located by SDS-PAGE, pooled (fraction II, 8 ml, 0.89 mg/ml), and then quickly applied to a 50 ml column of Biogel P-6DG equilibrated with 50 mM NaCl in buffer A (50 mM Tris-HCl (pH 7.8), 10 mM β-mercaptoethanol, 0.1 mM EDTA, 10 % (w/v) glycerol). Imidazole-free protein fractions (fraction III, 16 ml, 0.28 mg/ml), were applied to a 2.5 ml DEAE column equilibrated in buffer A plus 50 mM NaCl, and the flowthrough retained (fraction IV, 20 ml, 0.16 mg/ml). The protein in fraction IV was concentrated by either of two methods prior to storage. In some experiments fraction IV was simply dialysed against buffer A containing 50 mM NaCl plus 50 % (w/v) glycerol and then stored at −20 °C. Alternatively, fraction IV was applied to a 5 ml HiTrap heparin column equilibrated with buffer A plus 50 mM NaCl, and the protein eluted with 1 M NaCl in buffer A. Peak fractions were pooled, dialysed against buffer A containing 50 mM NaCl plus 50 % (w/v) glycerol, and stored at −20 °C (fraction V, 0.75 ml, 1.9 mg/ml). Fraction V had a specific topoisomerisation activity (Palaniyar *et al.*, 1996) of 431 units/μg. Mass spectrometry detected a single protein of mass 39,854 Da, in good agreement with the 39,874 Da predicted from the protein sequence.

E. coli ''vector control protein'' was extracted from pET21-transformed cells and eluted from a His-Bind column in a manner identical to SFV topoisomerase.

Telomere resolution reactions

A typical resolution reaction contained 2 μg of pSCB-1a substrate, 50 mM Tris-HCl (pH 7.5), and up to 5 μl of purified topoisomerase (or topoisomerase buffer) in 20 μl. Reactions were incubated at 37 °C for two hours and then chilled on ice. After adding NaCl to a final concentration of 1 M, the DNA was extracted with phenol-chloroform and precipitated in ethanol. The DNA was resuspended in 10 μl of water, 1.2 μl of 10× *Sca*I buffer plus 1 μl (10 units) of *Sca*I were added, and digested for another two to three hours at 37 °C prior to gel electrophoresis. *Sca*I cuts plasmid DNA poorly and even traces of residual contaminants from added proteins caused partial cleavage. Unfortunately it was the only enzyme which produced a simple pattern of well-resolved diagnostic restriction fragments. In an effort to avoid this difficulty, the DNA was treated with proteinase K and SDS, as described below, in an attempt to improve the efficiency of *Sca*I cleavage. However, some partial digestions occurred regardless of how the topoisomerase reaction was terminated and the DNA extracted (with salt and phenol, by heat inactivation at 80 °C, or with SDS/proteinase K and phenol).

Electrophoretic methods

Methods for the electrophoretic fractionation, visualisation, and quantification of DNA and protein are described elsewhere (Palaniyar *et al.*, 1996; Sambrook *et al.*, 1989). Alkaline agarose gels were fixed in 5 % trichloroacetic acid and partially dried prior to autoradiography. A Kodak density wedge and NIH Image software were used to correct for non-linearities in the response of image digitizers.

DNA end-labeling reactions

Enzymes were purchased from New England Biolabs and used as suggested with the supplied reaction components. To label hairpin reaction products (Figure 6), 10 μg of pSCB-1a was digested with 13 μg of fraction V topoisomerase in 0.5 ml of 50 mM Tris-HCl (pH 7.5) for two hours at 37 °C and then NaCl and SDS were added to final concentrations of 0.8 M and 0.5 % (w/v), respectively. The DNA was extracted with phenol, precipitated with isopropanol, and then digested for two hours at 37 °C with 50 units of *Ava*II in a 100 μl reaction. After heat inactivation of the *Ava*II, the DNA was end-labeled at 25 ° for 15 minutes using 5 units of Klenow polymerase and 50 μCi of [α-^{32}P]dCTP. Unincorporated nucleotides were removed using spin columns (Pharmacia S-300) and the DNA precipitated in ethanol in the presence of 20 μg of tRNA. pSCB-1a control DNAs were prepared in the same way except that DNA was cut with a combination of *Afl*II and *Ava*II and end-labeled in a reaction containing 25 μCi of [α-^{32}P]dCTP. Radiolabeled DNAs were fractionated on a 0.8 % agarose gel in Tris-acetate buffer, stained with ethidium bromide, and the 2.2 and 1.8 kb TIR-containing restriction fragments (Figure 1) excised and purified using a Gene Clean kit as directed by the manufacturer. *Bst*EII-cut lambda DNAs were end-labeled and column-purified in a similiar manner.

Other materials and methods

Radioisotopes were purchased from New England Nuclear and enzyme inhibitors from Sigma. Chromatography media were purchased from Novagen (His-Bind resin), Toyosoda (Toyopearl DEAE), BioRad (Biogel P-6DG), and Pharmacia (HiTrap heparin, Sephacryl S-300). Protein concentrations were determined using a dye-binding assay (BioRad) and bovine serum albumin as a standard.

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