

The circle is broken: telomere resolution in linear replicons

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Linear DNA molecules with covalently closed hairpin ends (telomeres) exist in a wide variety of organisms. Telomere resolution, a DNA breakage and reunion reaction in which replicated telomeres are processed into hairpin ends, is now known to be a common theme in poxviruses, *Borrelia burgdorferi* and *Escherichia coli* phage N15. Candidate proteins that may perform this reaction have recently been identified in poxviruses. Moreover, the first purification and definitive identification of a telomere resolvase has been reported for phage N15. This protein is the prototype for a new class of DNA enzyme that performs a unique reaction. Advances in the study of telomere resolution in poxviruses, *B. burgdorferi* and *E. coli* phage N15 are discussed.

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Abbreviations

lp17 linear 17 kb plasmid
ori origin of DNA replication
 SFV Shope fibroma virus

Introduction

The problem of replicating the 3' ends of linear template DNA molecules was first described in the 1970s [1]. Shortly thereafter, a model employing hairpin ends on linear DNA was proposed as a solution to the problem [2,3]. It is now well established that replication of the ends of linear DNA molecules can occur by several additional strategies, including telomerase-mediated extension of DNA ends, circularization or concatamerization to temporarily remove DNA ends, and protein-mediated priming at the ends of linear DNA molecules [4–6]. The molecular steps of some of these processes have now been described in some detail. However, progress in characterizing the replication of linear replicons with hairpin ends has lagged behind. Replicons with covalently closed hairpin ends have been described in a wide variety of organisms including poxviruses, African swine fever virus, *Chlorella* viruses, the *Escherichia coli* N15 prophage, certain mitochondrial DNAs, mitochondrial plasmids and plastid DNAs, and the chromosome and linear plasmids of bacteria in the genus *Borrelia* (which are spirochetes that cause Lyme disease and relapsing fever) [4,5,7–10]. In this review, we discuss recent advances in our understanding of the replication strategies employed in these systems. In particular, advances in the study of the DNA breakage and reunion reactions (telomere resolution) that process replication

intermediates into unit-length linear molecules with covalently closed hairpin termini in poxviruses, *Borrelia burgdorferi* and *E. coli* phage N15 are discussed.

Replication pathways for DNA with hairpin ends

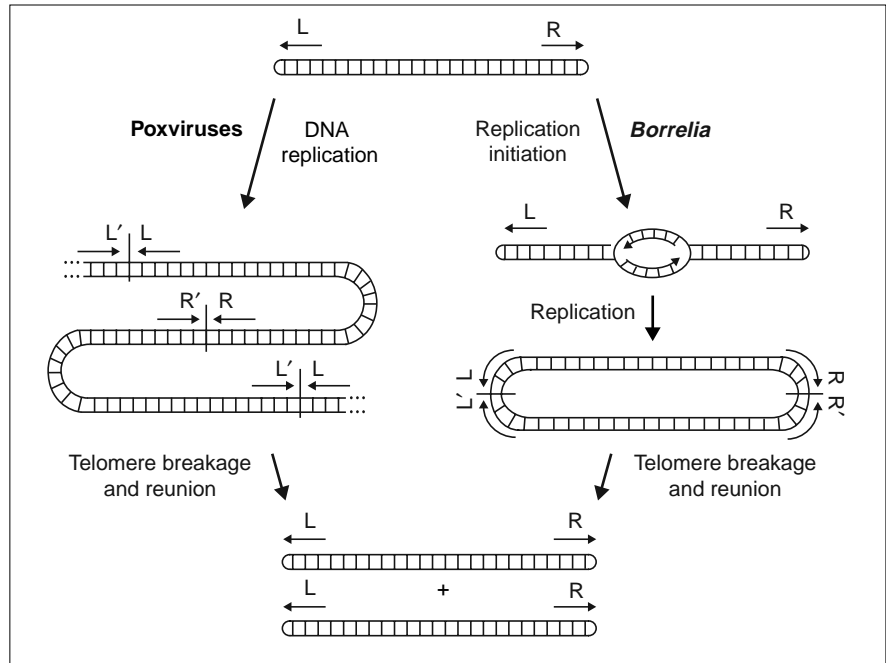
A number of models for replication of DNA molecules with hairpin telomeres have been described (see [4] for a recent review). Figure 1 shows the current state of thinking on the pathways used by poxviruses and *Borrelia*. In poxviruses such as vaccinia, the general pathway by which DNA replication occurs has been known for quite some time, but many of the molecular details have remained elusive. It is known that replication is initiated in the telomeric regions, resulting in the production of head-to-head and tail-to-tail concatameric arrays through strand displacement synthesis [11,12]. The dimer junctions (also referred to as replicated telomeres and depicted as L'–L and R–R' in Figure 1) subsequently serve as the sites of DNA breakage and reunion to regenerate unit-length molecules with covalently closed hairpin ends [13–15]. This process is referred to as telomere resolution and is directed by specific sequences in the telomeric regions. The enzyme that performs this process (the telomere resolvase) has not yet been definitively identified, although two candidate proteins have recently been reported and are discussed below.

In contrast to the poxviruses, for which the general scheme of DNA replication has been known for some time, studies on DNA replication in *Borrelia* have only been recently initiated. On the basis of the strand bias for transcription, the location of essential replication genes and the CG skew, the *ori* in the linear chromosome was suggested to be centrally located [16], rather than in the telomeric regions. Analysis of nascent DNA has recently enabled the first mapping of an origin of DNA replication (*ori*) in *Borrelia* to occur. A bidirectional *ori* was mapped (see Figure 1) to within a 2 kb region at the centre of the linear chromosome of *Borrelia burgdorferi* [17••]. Further sequence analysis predicted the *ori* to be located in a 240 bp stretch between the *dnaA* and *dnaN* genes, where a dramatic switch in CG skew occurs [17••]. Precise localization of the actual *ori* has been complicated by the absence of clearly definable DnaA boxes in the origin region.

An interesting feature of *B. burgdorferi* is that the genome is segmented, and in addition to the linear chromosome, there are about 12 linear and nine circular extrachromosomal elements [18•]. No plasmid origins have been mapped yet, but analysis of CG and AT skew suggests bidirectional replication from an internal origin, as found for the chromosome [19]. In spite of the apparent differences in the initiation of replication in poxviruses and *Borrelia*, recent work now appears to indicate that *Borrelia* also uses

Figure 1

Replication strategies for linear replicons with covalently closed hairpin ends in poxviruses and *Borrelia burgdorferi*. The L and R arrows indicate inverted repeats at the left and right ends, respectively. The line bisecting the head-to-head (L'-L) and tail-to-tail (R-R') telomere junctions in the replication intermediates is an axis of 180° rotational symmetry. This symmetry is perfect in *Borrelia* replicons but poxviruses have non-palindromic nucleotides and corresponding hairpins containing extra-helical bases. Replication of poxviral DNA is believed to be initiated by site-specific nicking in the telomeric regions followed by strand displacement synthesis from the nick site to give linear head-to-head or tail-to-tail dimers [11,12]. Additional rounds of replication would give rise to longer concatameric forms, which have been observed *in vivo* (see Figure 1 in [12]). In contrast, replication of the linear replicons in *Borrelia* is believed to start at internal bidirectional origins of replication [17**,19]. For both poxviruses and *Borrelia*, the replicated telomeres appear to be processed by a DNA breakage and reunion event (telomere resolution) to regenerate the covalently closed hairpin ends [13-15,20**]. Part of this figure is adapted, with permission, from [20**].



a telomere resolution step to regenerate monomeric molecules with hairpin ends [20**]. A replicated telomere from the left end of the linear 17 kb plasmid (lp17) in *B. burgdorferi* served as a target site for telomere resolution. A 140 bp or 70 bp replicated telomere was inserted internally into lp17 through a targeted recombination

event (see Figure 2). The internal site was an efficient substrate for telomere resolution *in vivo*, resulting in DNA breakage and reunion to generate a covalently closed hairpin telomere, as indicated in Figure 2. In contrast to previous findings for poxviral plasmids, autonomous replication of the transforming plasmid in *B. burgdorferi*

Figure 2

Telomere resolution in *B. burgdorferi*. A linear plasmid integrant carrying the replicated left-end telomere of lp17 was constructed using homologous recombination. The plasmid pGCL10-2, which carries the replicated telomere (L'-L, red), a kanamycin resistance gene (*kan*, blue), vector sequences (yellow) and an lp17 recombination target region (green) was used to transform *B. burgdorferi* to generate the indicated 21.6 kb lp17 derivative. The expected results from a telomere resolution event at the integrated L'-L site are shown. This figure is modified, with permission, from [20**].

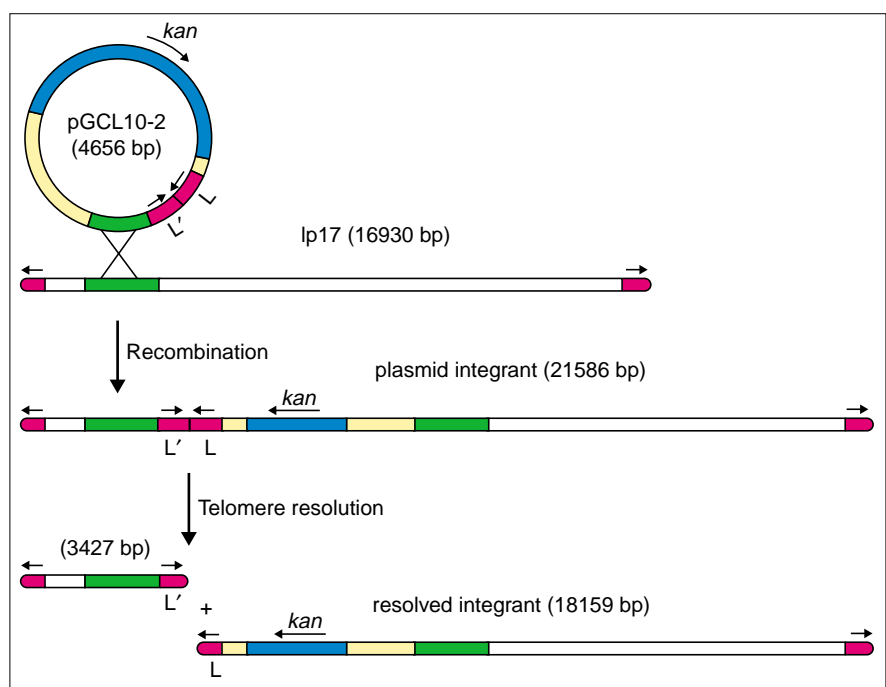
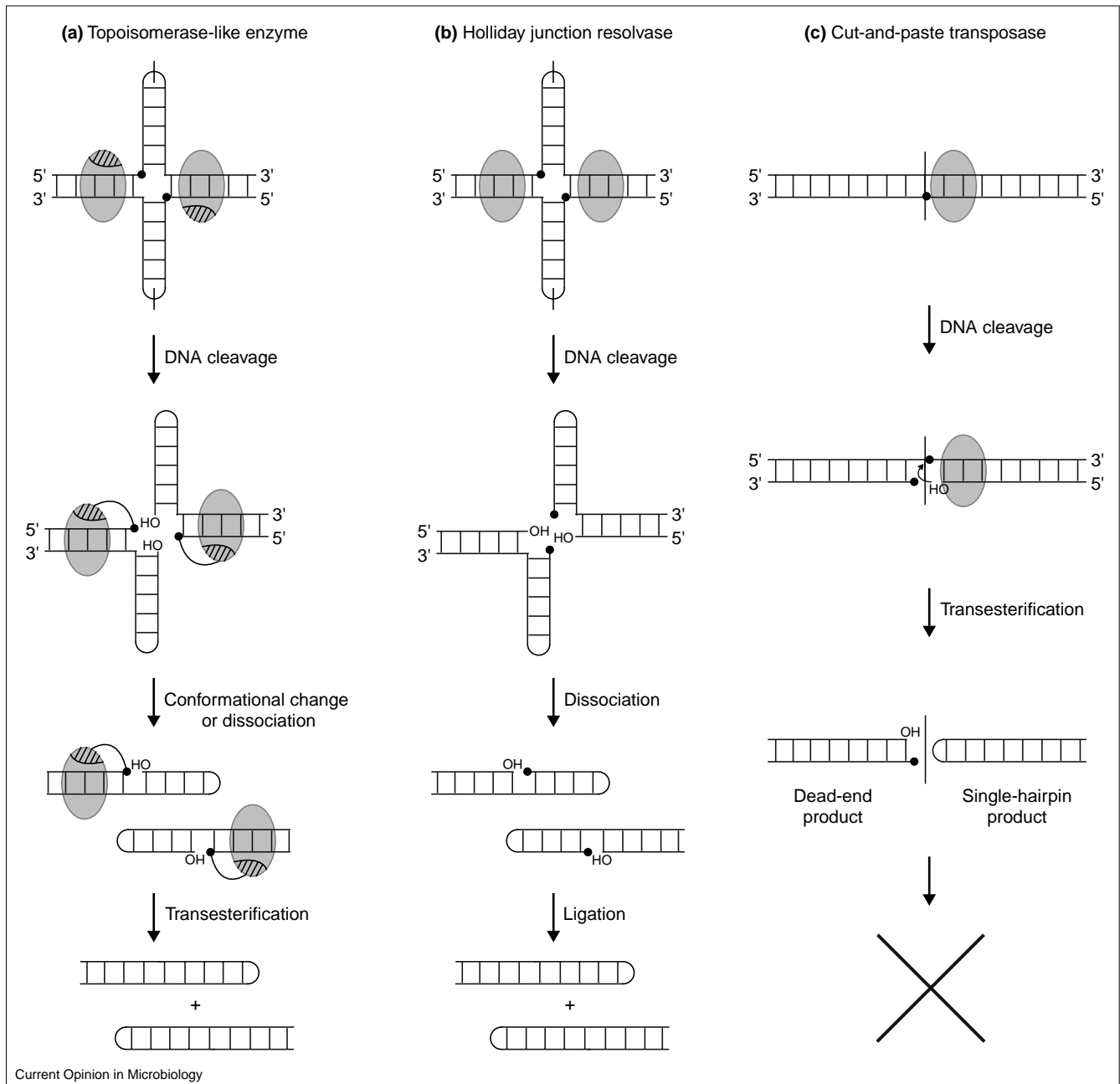


Figure 3 (legend opposite)



was not observed. Initiation of replication for lp17 therefore does not appear to utilize the telomeric regions carried on the plasmid, but likely involves an internal origin as for the *B. burgdorferi* chromosome. Moreover, direct insertion of the replicated telomere into a circular *E. coli*-*B. burgdorferi* shuttle vector [21•] resulted in conversion and maintenance of the circular replicon as a linear molecule [20••]. Telomere resolution in *B. burgdorferi* was also shown to be sequence-dependent. Substitution of the replicated telomere with an inverted repeat of the same size and base composition but with a different sequence to that of the *B. burgdorferi* telomere blocked

the resolution event. These results suggest that the telomere resolvase from *B. burgdorferi* recognizes a specific DNA sequence rather than an altered DNA structure resulting from the inverted-repeat structure of the replicated telomere. The telomere resolvase is therefore predicted to be a specialized protein rather than a non-specific Holliday junction resolvase (see mechanism section below). (A Holliday junction is a four-stranded DNA structure established by strand exchange between homologous DNA duplexes, and is a universal intermediate in homologous recombination.) The telomere resolution substrates characterized *in vivo* in this paper will be

Figure 3 legend

Possible mechanisms for telomere resolution. In (a,b), the inverted repeats present in a replicated telomere (see Figure 1) have been drawn as extruded cruciforms. (For discussions of cruciform extrusion in poxvirus DNA replication, see [12,15].) This isomerization is essential for the reaction mechanism shown in (b), but not for that depicted in (a). The lines bisecting the hairpin tips are the axes of symmetry in the replicated telomeres. The shaded ovals represent the telomere resolvase and the black dots denote phosphate groups involved in the reaction chemistry. In the pathway shown in (a), the general reaction mechanism of a Type IB topoisomerase-like enzyme [25,26] or a tyrosine recombinase [27–30] is detailed (although a Type IA topoisomerase or a serine recombinase reaction mechanism is also possible). Cleavage of the opposing DNA strands is effected by nucleophilic attack of the scissile phosphate from the hydroxyl group of an active site tyrosine (hatched region of the telomere resolvase). This results in the formation of a covalent protein–DNA intermediate that stores the energy of the broken bond in a 3′ phosphotyrosine linkage. A conformational change in the enzyme complex or simple dissociation of the two halves of the complex brings the free 5′ OH group on one

strand in proximity to the phosphotyrosine linkage on the opposite strand. The 5′ OH group attacks the linkage, resulting in a transesterification reaction to generate the closed hairpin products. No high-energy cofactor is required. In the pathway in (b), staggered DNA cleavages on opposing strands are introduced by endonucleolytic cleavage at the base of an extruded cruciform, as described for Holliday junction resolvases [31–36]. Dissociation of the complex brings the 5′ phosphate and the 3′ hydroxyl groups in proximity for ligase-mediated sealing of the nick to generate the closed hairpin products. A high-energy cofactor is required for all DNA ligases. In the pathway in (c), a cut-and-paste transposase [37–39] or V(D)J-like recombinase [40,41] reacts with the replicated telomere. These enzymes generate a hairpin intermediate. The 3′ OH group generated by cleavage of the first strand attacks the DNA backbone of the opposite strand to generate the hairpin. However, the reaction is self-limiting and results in a single hairpin product. A second hairpin cannot be generated because the 3′ OH needs to attack an intact phosphodiester bond to capture the energy required for hairpin formation. This mechanism is therefore not tenable for the production of two hairpin ends, as required for telomere resolution.

invaluable for purification and *in vitro* characterization of the telomere resolvase from *B. burgdorferi*.

Less is known about the replication of the temperate *E. coli* phage N15. It is a lambdoid phage that is maintained as a linear prophage with hairpin ends (see [8]). In spite of the lack of information about the general replication pathway of the N15 phage, it is the first system for which a functional telomere resolvase has been identified and purified [22••]. Because of its location adjacent to the left telomere, the TelN protein was originally predicted to form the hairpin telomeres of the phage (the genes for many phage-encoded proteins map near their site of action on the phage DNA). More recently, the TelN protein was found to have limited sequence homology to the HK022 integrase, a member of the tyrosine recombinase family that can break and rejoin DNA [8]. The protein has now been overexpressed and purified and has been shown to possess a DNA breakage and reunion activity that is active on a telomere junction from N15 [22••]. TelN alone regenerates the hairpin ends of N15 from either a supercoiled or linear substrate carrying a telomeric junction.

Before proceeding to a discussion of mechanistic issues of telomere resolution, we would like to comment on the nomenclature for telomere-resolving enzymes. The predicted N15 protein TelN was originally termed a ‘telomerase’. This name was subsequently changed to ‘protelomerase’, for ‘prokaryotic telomerase’ [8]. Nonetheless, the purified protein does not demonstrate telomerase activity. Moreover, as noted above, telomere resolution is not limited to prokaryotic organisms and was, in fact, discovered in the eukaryotic poxviruses. It therefore seems most appropriate to use a more descriptive and mechanistically correct name. The term ‘telomere resolvase’, which has appeared in the poxvirus literature [23••,24•], fulfills both of these criteria.

Molecular mechanisms for telomere resolution

There are two reaction mechanisms by which telomere resolution may proceed; these are depicted in Figure 3ab. In the topoisomerase-like mechanism (Figure 3a), a high-energy cofactor is not required. Instead, phosphodiester bond energy is conserved through the formation, upon DNA cleavage, of an intermediate of a covalent protein–DNA reaction. The subsequent transesterification step then forms a link between nucleotides that were originally situated on opposite DNA strands, generating linear molecules with covalently closed hairpin ends. A single enzyme performs both chemical steps of the reaction. This type of reaction mechanism is used by both topoisomerases [25,26] and site-specific recombinases [27–30].

In contrast, the Holliday junction resolvase mechanism requires a high-energy cofactor, as phosphodiester bond energy is not conserved (Figure 3b). The cleavage step of this reaction is mediated by a four-way-junction-resolving enzyme typified by the *E. coli* RuvC protein [31,32]. An extruded cruciform has the essential features of a Holliday junction recombination intermediate that are required for recognition and processing by a junction-resolving enzyme [33–36]. Following DNA cleavage, joining by DNA ligase of nucleotides that were originally situated on opposite DNA strands results in linear products with covalently closed hairpin ends.

Finally, Figure 3c shows the mechanism used by cut-and-paste transposases [37,38] to generate covalently closed hairpin ends that are an intermediate in transposition [37–39] and V(D)J recombination (a process in which immunoglobulin genes are assembled in developing lymphocytes) [40,41]. Although this reaction mechanism is now well established for hairpin formation in non-replicative transposition and V(D)J joining, it is not plausible for telomere resolution — because of the energetic constraints

of the reaction, only a single hairpin can be generated, as described in the legend to Figure 3.

Telomere resolvases

In recent years, two different candidate proteins for telomere resolution using the mechanisms in Figures 3a,b in poxviruses have been described. For reasons discussed elsewhere [15,42], the poxviral resolution reaction is thought to occur on a cruciform structure, which, like recombination intermediates, contains a four-way junction. In addition to its other well characterized activities, recombinant poxvirus (vaccinia virus) topoisomerase has been shown to be capable of functioning as a Holliday junction resolvase [24•,43]. Vaccinia topoisomerase cleaves at two 5' CCCTT↓ sites (the arrow denotes DNA cleavage) when they are opposed in a partially mobile, synthetic four-way junction (see Figure 3a). The subsequent transesterification results in two linear duplex DNAs. Studies with poxviral topoisomerases were taken a step further using a recombinant topoisomerase from Shope fibroma virus (SFV). *In vitro* reactions were performed with plasmids carrying replicated SFV telomeres that had previously been shown to function as *in vivo* resolution substrates [23••]. A supercoiled plasmid with 1.5 kb of replicated telomere was resolved into a linear molecule with covalently closed hairpin termini. The authors demonstrated a need for the telomere junction to be extruded as a cruciform. In addition, branch migration was required for the reaction, as inhibition of this process abolished the telomere resolution activity of the topoisomerase. The inefficiency of the *in vitro* plasmid reaction and the disparity between the activity of different replicated telomeres *in vivo* versus *in vitro* with SFV topoisomerase suggests that the topoisomerase may not be the real telomere resolvase. These studies have nonetheless established the ability of the viral topoisomerase to perform telomere resolution to yield authentic products with covalently closed hairpin ends, as depicted in Figure 3a.

A second candidate protein for the poxviral telomere resolvase was identified by sequence gazing using a new generation of sequence similarity software (PSI-BLAST [44]; http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-psi_blast). Garcia *et al.* [45•] uncovered a RuvC analogue present in all sequenced poxviruses, the first demonstration of such an enzyme in metazoans or their viruses. They verified that a recombinant version of the vaccinia A22R protein was indeed a Holliday junction resolvase. Sequence specificity or activity on a replicated poxviral telomere has yet to be established, but this work clearly raises the possibility that A22R is involved in telomere resolution in poxviruses that use the mechanism depicted in Figure 3b. More recently, analysis of conditional lethal A22R mutants showed that late-stage DNA replication was reduced and that resolution of telomere junctions was severely delayed, but not eliminated [46•]. A similar analysis with conditional lethal mutations in the poxviral topoisomerase would also be helpful in sorting out the real telomere resolvase, as would an explanation for why temperature-sensitive mutations in

the RNA capping machinery cause a defect in telomere resolution [47,48]. The possibility has not yet been completely ruled out that a more highly specialized enzyme performs telomere resolution in poxviruses.

The prototype for such a specialized telomere resolvase has been recently reported for the *E. coli* phage N15 [22••]. The purified 630-residue N15 TelN protein efficiently resolves N15 telomere junctions into covalently closed hairpins. A high-energy cofactor is not required for the reaction. Furthermore, telomere resolution by TelN was equally efficient on supercoiled or linear substrates, suggesting that cruciform formation in the substrate is not required for the reaction. The TelN protein has limited sequence homology to conserved residues in the active site region of the integrase family of site-specific recombinases, also known as tyrosine recombinases [8]. These enzymes employ an active-site tyrosine and a reaction mechanism using a 3'phosphotyrosine linkage in a covalent intermediate [27,28,30] also characteristic of the Type IB topoisomerases [25,26], as shown in Figure 3a. The TelN reaction almost certainly proceeds through this pathway or some variation of it. A covalent protein–DNA intermediate has not yet been reported, but the N15 telomere resolvase loses all activity when the putative active site tyrosine is changed to a phenylalanine [22••].

Although the mechanism of breakage and joining used by TelN appears to mimic that previously observed for Type IB topoisomerases and tyrosine recombinases, we would like to emphasize that the telomere resolution activity displayed by TelN represents a unique reaction. Type IB topoisomerases promote breakage and reunion of a single DNA strand to alter the topological state of a DNA molecule. Site-specific recombinases perform a more complex event in which four DNA strands are broken and subsequently joined to a different DNA duplex, resulting in the production of a recombinant product. The N15 telomere resolvase must break two phosphodiester bonds in a single DNA duplex (one on each strand) and subsequently join each end with the opposite DNA strand to form a covalently closed hairpin.

Telomere resolution and genome plasticity

Recent sequence analysis of *B. burgdorferi* DNA has indicated a fluid genome with widespread DNA rearrangements. Of particular interest to the work reviewed above is the high degree of sequence similarity that can be found in regions within several kilobases of the telomeres on many *B. burgdorferi* replicons [18•,49]. These duplications may be related to the increased homologous recombination specifically associated with transformation of functional *B. burgdorferi* telomeres [20••]. DNA breakage events during telomere resolution may be involved in stimulating not only homologous recombination, but illegitimate recombination as well, with a resulting exchange of genetic information in DNA regions near the telomeres.

Conclusions

The process of telomere resolution is an integral part of the replication of linear DNA molecules with hairpin ends from phage, poxviruses and *Borrelia*. Two candidate proteins for the telomere resolvase have been reported in vaccinia virus and SFV. A substrate for telomere resolution has been constructed and tested in *B. burgdorferi* and should prove invaluable for *in vitro* assays to purify the telomere resolvase from *B. burgdorferi*. Finally, the first conclusively identified telomere resolvase has been purified from the *E. coli* phage N15. The enzyme appears to function through a reaction mechanism similar to that of Type IB topoisomerases and tyrosine recombinases, but performs a unique reaction to generate covalently closed hairpin ends from a telomere junction. Future studies on poxviruses, *Borrelia* and other organisms will reveal whether or not a unity exists in the telomere resolution mechanism of widely divergent organisms.

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