Phage N15 Telomere Resolution

TARGET REQUIREMENTS FOR RECOGNITION AND PROCESSING BY THE PROTELOMERASE

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The Escherichia coli prophage N15 exists as a linear DNA molecule with covalently closed ends. Purified N15 protelomerase TelN is the only protein required to convert circular DNA substrates to the linear form with hairpin termini. Within the context of the telomere occupancy site tos, the target for TelN is the 56-bp telRL consisting of the central 22-bp palindromic telO and two 14-bp flanking inverted sequence repetitions. DNase I footprinting of TelN-telRL complexes shows a segment of ~50 bp protected by TelN. Surface plasmon resonance studies demonstrate that this extended footprint is caused by two TelN molecules bound to telRL. Stable TelN-target DNA complexes are achieved with telRL; however, the additional sequences of tos stabilize the TelN-target complexes. TelO alone is not sufficient for specific stable complex formation. However, processing can occur, i.e. generation of the linear covalently closed DNA. Within the context of telRL, sequences of telO are involved in specific TelN-telRL complex formation, in processing itself, and/or in recognition of the processing site. The sequence of the central (CG)3 within telO that is part of a 14-bp stretch proposed to have Z-DNA conformation is essential for processing but not for formation of specific TelN-telRL complexes. The concerted action of both TelN molecules at the target site is the basis for telomere resolution. Capturing of reaction intermediates demonstrates that TelN binds covalently to the 3'-phosphoryl of the cleaved strands.

N15 is a lambdoid, temperate Escherichia coli phage that has double-stranded DNA with cohesive ends packaged in the virion (1). The complete nucleotide sequence of the 46-kb genome is known (2). Upon infection of E. coli, the DNA circularizes via the cos ends. In contrast to other temperate phages, N15 does not integrate into the host genome. Lysogeny is established by processing the telomere resolution site telRL to form a linear genome with covalently closed, hairpin-like ends telL and telR (see “Discussion”). The proposed telomerase occupancy site tos (1) contains a series of inverted repeats centered on the 56-bp palindromic telRL. This palindrome or part of it could form a potential cruciform structure that may function as the real substrate of the telomerase TelN. In vivo replication of linear N15 plasmids requires three N15-derived components as follows: the origin of replication ori, the replication initiation protein RepA, and TelN protein for resolution of the replicated telomeres (3). Ori probably is located immediately upstream of repA. RepA is essential in N15 lytic replication and for maintenance of the linear prophage. Both lytic and lysogenic replication is independent of at least dnaA, dnaJ, dnaK, grpE, and recA (1, 4, 5). For telomere resolution, in vitro purified TelN and a telRL substrate are sufficient (6). In contrast to eukaryotic telomerases, TelN seems to be purely proteinaceous, lacking DNA synthesizing activity. The cleaving-joining activity of TelN resembles a specialized type of integrase that uses the same DNA molecule as donor and as acceptor.

Phage N15 belongs to the small group of systems known to replicate as linear DNA with hairpin ends. Such linear genomes are generally of eukaryotic origin, but a few from eubacteria are known: Yersinia bacteriophage PY54; a Klebsiella plasmid (7); the plasmids and genomes of Borrelia (8, 9) and Agrobacterium tumefaciens (10, 11); poxviruses (e.g. vaccinia virus (12)), iridopoxvirus (e.g. African swine fever virus (13)), and parvovirus (e.g. minute virus of mice (14)); and the genomes and certain plasmids of mitochondria and plastids (15–17). In none of these cases is replication clearly understood. However, several models for replication of linear DNA with hairpin ends exist (18). In one group, initiation of replication occurs at the hairpin ends by site-specific cleavage of one strand within the hairpin ends. The 3'-hydroxyls are then used as primers for strand displacement DNA synthesis. The other group contains an internal ori where θ-type replication initiates bidirectionally resulting in circular head-to-head and tail-to-tail dimers. These intermediates would then be processed by a cleaving-joining reaction, the telomerase resolution, to regenerate covalently closed hairpin ends in the progeny molecules. Recently, telomere resolution was demonstrated in vivo for the linear Borrelia burgdorferi plasmid lp17 using telomere substrates (19). For phage N15, even in vivo circular dimeric replication intermediates, resolution of these molecules via TelN telomerase activity was described (3). This demonstrates that one completed round of replication results in two progeny molecules as is the case for most replication mechanisms except the rolling circle type.

Phage N15 serves as a model system to study replication of linear DNA with hairpin ends that allows the use of E. coli extracts circumventing pathogenic organisms or cells that are difficult to grow. This is the first report describing the architecture of the complex of a telomerase resolvase with the cognate telomere resolution site, the nucleotide sequence requirements for target recognition and processing, and the properties of a captured reaction intermediate. We unravel binding of the protelomerase TelN to the target using fragment retention on gels and

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§ S. Hertwig, I. Klein, R. Lurz, E. Lanka, and B. Appel, unpublished data.
DNase I footprinting combined with surface plasmon resonance and mutagenesis studies.

**EXPERIMENTAL PROCEDURES**

*DNA Fragmentation and Stability Assays—* coli SCS1 (recA1, endA1, gyrA-96, thi-1, hisd17(r. m.), supE44, relA1, StraguA) was used as the host for the telN overexpression plasmids and the telRL target structures (6). SURF 2 (e1: MerA), ΔmercB-bsdHSM-rmyr171, endA1, supE44, thi-1, gyrA96, relA1, lac, recB, recF, shcC, umacC:TrsK5(Km)), were linearized with either Dra I, PvuII, or SfiI. The resulting double-stranded fragment was incubated with 1 μg of TeIN target sequence (6). Recombinant plasmids used in this study are described in Table I. Cells were grown as described previously (20). When appropriate, antibiotics were added to the following concentrations: ampicillin (sodium salt, 100 μg/ml), tetracycline-HCl (10 μg/ml), or kanamycin sulfate (30 μg/ml).

**Overexpression and Purification of Proteins—** TeIN wild type protein was overproduced and purified using the procedure described previously (6). The same procedure was used to purify TeIN Y424F to near-homogeneity. In both cases fraction V (6) was used.

**DNA Techniques—** Standard techniques for DNA manipulation were used (21). The nucleotide sequence of recombinant DNA was verified by the dideoxy chain termination method (22).

**Construction of Plasmids—** By using synthetic oligonucleotides, we constructed the plasmids pJD105-Sym, pJD105A20T21A, pJD105A-T-sym, pJD105(TA), pJD105(CG)1, pJD105SS, pJD105A8, pJD105ΔS.2 and pJD105ΔS.7, which all carry a telRL derivative (Table II). The oligonucleotides were annealed to form the telRL region with the desired mutation or deletion and HindIII and BamHI restriction sites at the fragment ends. The resulting double-stranded fragment was inserted as HindIII-BamHI fragment into pBR329 that had been prepared accordingly (6). The pBR329 vector fragment was then religated without any insert, to form pBR329Δ1. The plasmid pJD101Y424F was constructed using PCR-based mutagenesis as described (23) with synthetic primers (Table II) and pJD101 as template. The construction of several plasmids used in this study was described previously (Table I).

The nucleotide sequence of all DNA fragments inserted into vector plasmids was confirmed (see above).

**Transmission Electron Microscopy—** For mapping the binding sites of TeIN by electron microscopy, the plasmids containing various TelN targets, pJD104 (harboring tos), pJD105 (telRL), or pJD106 (telO) (6), were linearized with either XbaI or Asel. In the case of pJD104 SphI was used instead of Asel. The linearized DNA was purified by electrophoresis on 0.7% agarose gels and subsequent gel extraction from the agarose slab. The plasmid was then incubated with 1–3 pmol of TeIN Y424F protein in 10 μl of buffer containing 20 mM Tris-HCl (pH 7.6), 25 mM potassium glutamate, 0.1 mM EDTA, 1 mM dithiothreitol, 5 μM of PCR fragment was incubated with 0–50 pmol of TeIN protein for 30 min at 30 °C. DNAase I cleavage reactions were performed essentially as described (29, 30). 1.5 μl of DNAase I (0.0015 units, RNase-free, Roche Molecular Biochemicals) was added to the TeIN-DNA complexes. Following 10 min at 37 °C, the DNAse I was inactivated by adding 50 μl of a mixture of 1% SDS, 200 mM NaCl, 20 mM EDTA (pH 8.0), 25 μg/ml salmon sperm DNA. The reaction products were phenol-extracted and precipitated with ethanol containing 100 μg/ml glycerol. The pellet was dissolved in 5 μl of buffer (40 mM Tris-HCl (pH 8.8), 2 mM EDTA), mixed with 2 μl of formamide/methylene glycol, heated for 3 min at 95 °C, and loaded onto a 6% (w/v) polyacrylamide-urea sequencing gel (30). The cleavage pattern was visualized by autoradiography as described for the fragment retention assay. To generate a fragment pattern for the nucleotide sequence, dideoxy cycle sequencing was performed using the same conditions, templates, and radiolabeled primers as for the generation of the PCR fragments, except that Vent (exo−) polymerase was used as described (31).

**Surface Plasmon Resonance Studies—** Surface plasmon resonance was determined using a Bioscan 2000 instrument (Biacore AB). Two DNA fragments were formed using synthetic oligonucleotides (Table II). One fragment consists of telRL within the original N15 nucleotide context, whereas the sequence of the other one was random. The DNA fragments contained telRL R2/L2 flanking sequences and were immobilized on the sensor chip, as described previously. The DNA fragments were immobilized at a density of 3–5 ng/ml, and an RU signal was recorded. RU of each fragment immobilized at one end was immobilized at the streptavidin matrix in separate chambers of an SA sensor chip according to the manufacturer’s recommendations. 250 μl of TeIN Y424F in various concentrations in buffer consisting of 20 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 50 μg/ml bovine serum albumin, 0.005% P2 surfactant (Biacore), or without calf thymus DNA were injected for 375 s over the immobilized DNA. Then the complexes formed were washed with 120 μl of the same buffer for 180 s to allow for dissociation. The flow rate always was 40 μl/min. All runs were performed at 25 °C. Changes in plasmon resonance correspond to a change in mass bound to the chip and are measured in resonance units (1 RU corresponds to 1 ng/ml for proteins). Resonance response for protein is higher than that observed for DNA (32). For double-stranded DNA it was reported that RUprotein is increased by 0.73 RU DNA (33). Therefore, the values measured were corrected using this factor. Data were evaluated according to Ref. 33 using the BIA-evaluate software version 3.0 (Biacore AB).

**RESULTS**

*The TelIN-binding Site in tos Is telRL—* Telomere resolution requires recognition and binding to the target DNA by TeIN before processing via transelaboration could take place. To study the recognition and binding step separated from telomere resolution, it was necessary to uncouple binding and transelaboration. Hence, if not otherwise stated, for all protein-DNA binding studies a mutant TeIN protein was used, in which the proposed active site Tyr-424 was replaced by a phenylalanine (6). TeIN Y424F has lost its transelaboration activity but retained its specific DNA binding ability. To locate the binding site of TeIN on the DNA, complex formation with three substrates, tos, telRL, and telO, was followed by electron microscopy. Tos (telomerase occupancy site) has a size of 310 bp and is defined by the inverted sequence repetitions R1/L1 and R2/L2 flanking telRL (1). The repeats R3/L3 enclose the palindromic telomerase resolution site telOR forming telRL (56 bp). The tos substrate used here consists of 563 bp including neighboring N15-derived base pairs (6) (Table I). Knobs on the DNA indicate likely representative examples of complexes of TelN with tos (Fig. 1). Plotting the positions of the knobs versus the contour length of the DNA yielded histograms demonstrating

2The abbreviation used is: RU, resonance units.
the specificity of binding. As indicated by the peak positions in the histograms, site-specific complex formation was detected with tos and telRL only (Fig. 2, A and B). The positions of the dots in TelN-telO complexes were scattered randomly along the DNA and did not map preferentially to a unique region showing that these complexes arose from non-sequence specific binding (Fig. 2C). From these data we conclude that tos has a unique binding site for TelN. Because telRL (56 bp) alone is sufficient for binding and the positions of the peaks on tos and telRL match perfectly, this binding site is telRL. TelRL consists of two inverted sequence repetitions, R3 and L3 (14 bp each), and the central palindromic telO (22 bp). The data on TelN-telRL (Fig. 2B) and TelN-telO (Fig. 2C) complexes show in addition that the sequences R3 and L3 are essential for specific complex formation, because telO alone failed to yield specific complexes. To learn more on the specificity of binding, the complex formation was studied by fragment retention in gel electrophoresis.

TelO no specific binding was detected (Fig. 3A, lanes j–l) confirming the data obtained by electron microscopy (Fig. 2C). The clear indication for nonspecific binding in the telO and vector experiment is that the $K_d$ values of fragment D with and without telO are identical (1300 nM; Fig. 3B and Table III). Furthermore, the largest band alters its position first, and upon increase of the protein amount each of the fragments migrates slower. To answer the question if binding of wild type and mutant TelN is comparable, we used a non-processed telRL derivative (telRLA20TT21A, Fig. 3A, lanes g–i). The almost identical dissociation constants of 290 and 310 nM (Fig. 3B and Table III) demonstrate that both proteins TelN and TelNY424F bind telRLA20TT21A with similar affinities indicating that the TelN Tyr to Phe mutation does not influence binding dramatically. Interestingly, a more than 2-fold difference of the $K_d$ values was observed in comparison to telRL. The TelN-telRL complex was apparently more stable than TelN/telRLA20TT21A. Thus the difference in stability is probably because of the AT to TA mutation. The most stable complexes were formed with tos ($K_{d(app)} = 25$ nM). There is an almost 5-fold increase in stability of the TelN-tos complexes compared with telRL complexes ($K_{d(app)} = 130$ nM; Table III) suggesting that the additional invert sequence repetitions, R1/L1 and R2/L2 present in tos considerably contribute to this effect. It should be kept in mind that TelN has a high affinity to DNA in general, i.e. it binds DNA in a non-sequence-specific fashion. To obtain preliminary data on the protein-complex architecture we used DNase I footprinting.

TelN Covers a 50-bp Stretch of telRL—DNase I footprints on TelN-telRL complexes demonstrate that a large stretch of nu-
cleotides is protected against nuclease attack on both strands (Fig. 4). The protection pattern suggests that ~50 bp are inaccessible to DNase I cleavage. The protection pattern shows one hypersensitive band on each strand. The footprint of the upper strand is divided in alternating stronger and weaker protected segments. This pattern is only rudimentary on the lower strand. Mapping the data onto the telRL sequence demonstrates a mirror symmetric arrangement of the hypersensitive bands (Fig. 5) separating two regions, one of 52 bp and another of 6 bp. To determine the binding stoichiometry of TelN-telRL complexes that might partially explain the extended binding of 6 bp, TelN Y424F was used. The histograms were generated by determining the frequency of distribution of TelN protein bound (DNA/protein = 1:37) over 1000 equidistant points on the target DNA, counting all protein complexes bound within a 2% (~80 bp) window centered on each point. To show that TelN has only one unique binding site, the DNA restriction maps of the plasmids were matched and superimposed on each other. The vertical hatched line depicts the symmetry center of the telRL, and telO. The lines (red and blue) above each histogram represent the whole length of the plasmid for each type of linearization. Relevant restriction sites are marked. The black box within the histogram represents the N15 insert. A depicts pJD104 (4374 bp) linearized with SphI (blue, 114 molecules with protein bound were analyzed and aligned) and AatII (red, 178 molecules). B, pJD105 (3867 bp) linearized with AseI (297 molecules) and AatII (188 molecules). C, pJD106 (3831 bp) with AseI (164) and AatII (111 molecules). The left and the right axes of the panels both give the number of the complexes measured. The bar represents 500 bp.

Site—Previous studies (6) by glycerol gradient centrifugation indicated that TelN protein is likely to exist as a monomer in solution. To verify this result by a different approach, we used surface plasmon resonance. TelN protein was covalently bound to a B1 chip designed for protein attachment. After extensive washing at high salt concentration (1 M NaCl) to dissociate eventually the present oligomers, the TelN solution was passed over the TelN-loaded chip. Almost no mass increase was detected indicating that TelN/TelN interaction does not take place under the conditions used (data not shown). This finding confirms that TelN indeed is monomeric in solution. Hence the question was asked what happens to the stoichiometry of TelN binding if the target DNA telRL is present. The telRL DNA used in the binding experiments was 97 bp long, i.e. the 56-bp sequence of telRL was flanked by 18/23 nucleotides of N15 DNA.

Two TelN Monomers Interact with the Telomere Resolution Site

Fig. 2. The binding site of TelN within telRL. Each of the plasmids applied was linearized in separate experiments with two restriction enzymes, each cutting the DNA only once. For electron microscopy, the protein-DNA complexes were formed as given under “Experimental Procedures.” TelN Y424F was used. The histograms were generated by determining the frequency of distribution of TelN protein bound (DNA/protein = 1:37) over 1000 equidistant points on the target DNA, counting all protein complexes bound within a 2% (~80 bp) window centered on each point. To show that TelN has only one unique binding site, the DNA restriction maps of the plasmids were matched and superimposed on each other. The vertical hatched line depicts the symmetry center of the telRL, and telO. The lines (red and blue) above each histogram represent the whole length of the plasmid for each type of linearization. Relevant restriction sites are marked. The black box within the histogram represents the N15 insert. A depicts pJD104 (4374 bp) linearized with SphI (blue, 114 molecules with protein bound were analyzed and aligned) and AatII (red, 178 molecules). B, pJD105 (3867 bp) linearized with AseI (297 molecules) and AatII (188 molecules). C, pJD106 (3831 bp) with AseI (164) and AatII (111 molecules). The left and the right axes of the panels both give the number of the complexes measured. The bar represents 500 bp.

Fig. 3. A, representative autoradiographs of DNA fragment retention on gels demonstrate specific binding of telRL and telO by TelN Y424F. The experiments were carried out as described under “Experimental Procedures.” Fragment D contains the TelN target structure. The other fragments serve as competitor DNA. Lanes a–c contain fragments from pJD104 (fragment D, telRL, 510 bp) incubated at 0, 0.05, and 0.15 μM protein; lanes d–f, pJD105 (fragment D, telRL, 517 bp) at 0, 0.1, and 0.2 μM TelN; lanes g–i, pJD105 A20TT21A (fragment D, telRL, A20TT21A, 517 bp) at 0, 0.4, and 0.6 μM TelN; lanes j–l, pBR322A1 (fragment A, no N15 DNA, 463 bp) at 0, 1.0, and 1.8 μM TelN. A, equilibrium binding of telRL, and telO to TelN Y424F. Fragment D from A was quantified as described under “Experimental Procedures.” TelN concentrations from 0 to 2.5 μM were used. For each of the plasmids, the values plotted were averaged from at least two independent experiments. The apparent equilibrium dissociation constant Kd(app) for telRL A20TT21A was determined using both TelN wt protein and TelN Y424F, respectively.
to facilitate access of TelN to its target. A random DNA sequence of the same length served as reference for baseline adjustment. The highest value determined was 450 RU corresponding to 1.64 TelN molecules per telRL target (Fig. 6, lane k). We interpret the overlay sequence repetition also abolished the sequence-specific interaction of this truncated telRL substrate (Fig. 7, pDBI0518). These observations indicate that the inverted repeat sequences led to a gradual loss of the specific DNA binding ability. At the substrate size of 56 bp specific binding was no longer observed (data not shown). The complete removal of one arm (R3) of the inverted sequence repetition abolished the sequence-specific interaction of this truncated telRL-substrate (Fig. 7).

Fig. 5. Schematic representation of TelN-protected nucleotides of telRL. The nucleotide sequence of telRL containing either telRL or a random nucleotide sequence (Table II) immobilized in different chambers of the sensor chip. After injection of 250 μl of TelN Y424F at a flow rate of 40 μl/min, the chip was washed with 120 μl of buffer to allow for dissociation of the telRL-TelN complexes. For details see “Experimental Procedures.” Overlays of representative sensorgrams obtained for a variety of TelN concentrations are shown. The protein concentrations used are given at right-hand side of both panels. A, sensorgrams of TelN Y424F without competitor DNA. B, sensorgrams of TelN Y424F with calf thymus DNA added as competitor DNA. The ratio TelN:competitor is identical for each of the protein concentrations used.

Affinity of telRL for TelN Is Modulated by the Repeats R3/L3—Reduction in binding affinity was already observed when toc (Table III, 28 nM) was truncated to telRL (Kd(app) = 130 nM) by the removal of R1/L1 and R2/L2. Symmetric step-wise reduction of the length of the 56-bp telRL sequence led to a gradual loss of the specific DNA binding ability. At the substrate size of 36-bp specific binding was no longer observed (data not shown). The complete removal of one arm (R3) of the inverted sequence repetition also abolished the sequence-specific interaction of this truncated telRL substrate (Fig. 7, pDBI0518). These observations indicate that the inverted repeats R1/L1, R2/L2, and also R3/L3 are responsible for increasing the binding affinity to the telomere resolution site telO. The introduction of symmetry into telRL (pJD105R-sym) outside of telO at the 4 bp (15, 17, 40, and 42, see Fig. 5), which are the
basis for the original asymmetry in the otherwise palindromic telRL sequence, does not have any significant influence on binding or processing (Fig. 7). Consequently, further analysis concentrated on the substrate properties of telO.

TelO Is Sufficient for Telomere Resolution—Binding of telO by TelN occurs at the level of nonspecific interactions (Fig. 2C, Table III, and Fig. 7). Therefore, no telomere resolution was detected at TelN concentrations applied for telRL processing. However, at TelN concentrations of ~50-fold higher than used for telRL, the telO substrate (pJD106) is processed weakly indicating that telO contains all sequence requirements needed for telomere resolution in vitro. Because of the lack of specific binding of telO the question of what is needed for telO recognition and processing in terms of nucleotide sequence was addressed by using mutations within telO in the context of telRL (Fig. 7).

TelO Is Essential for Specific Recognition and Processing—Binding and processing studies were initiated with telRLA-20TTT21A, a target mutation containing an asymmetric sequence situation in telO (see above and Fig. 7). This derivative formed less stable complexes with wild type TelN than wild type telRL (Table III), and processing could not take place anymore (Fig. 7). These data already indicated that the sequence in the central palindrome is of major importance in telomere resolution. Restoring symmetry in the A20TTT21A mutation, by introducing A36TT37A, reduced specific binding further indicating that telO apparently is involved in recognition and binding. Not surprisingly, the nucleotide sequence also determines whether processing could occur or not. The telO core sequence (20 bp) most likely lies between bases pairs 19 and 38 because derivative pJD105Δ8 still was processed but pJD105Δ8.1, 1 bp shorter on each side, was not. Replacement of part of the core sequence, proposed to have 2-DNA conformation (1) by a symmetric but otherwise random sequence (pJD105S, Table II) was inert in binding and processing. Extension of the central (CG)3 to (CG)5 by symmetric replacement of the flanking TATA region by CGCG also proved to behave inertly in binding, recognition, and processing (pJD105(CG)5; see Fig. 7). Surprisingly, the replacement of the central (CG)3 by TATATA (pJD105[TA]3) retained full binding capacity, but processing of this derivative was considerably altered.

Processing of the circular (TA)7 substrate by TelN yielded several diffuse bands upon agarose gel electrophoresis, the characters of which were not readily interpreted. Inspection of the sample by electron microscopy indicated that the majority of the DNA consisted of circular molecules decorated with at least one dot, each most likely representing a covalently attached TelN molecule. The TelN-DNA molecules may represent intermediates of the resolution reaction (data not shown). Processing of linear (TA)7 substrate, i.e., linearized by cleavage with the restriction enzyme NruI prior to the TelN reaction, yielded two clear sharp bands of the expected size when treated with proteinase K before electrophoresis (data not shown). The finding indicated that processing of the (TA)7 substrate occurred at or around the predicted telomere resolution site. The larger of the two fragments was selected for further analysis by end labeling.

TelN Binds Covalently to the 3'-Ends of the Telomere Resolution Site—Labeling of the 3'-end or the 5'-end demonstrates free accessibility, i.e. a protein bound to the respective end would prevent attachment of the label. Linearization of the (TA)7 substrate pJD105/T(A)7 and subsequent processing with TelN yielded two fragments. The longer one (3239 bp) was isolated and 32P-labeled at the 3'-ends or the 5'-ends. If TelN is attached to the 3'-end, only one side of this fragment is expected to be 3'-labeled, whereas both 5'-ends should carry the label (Fig. 8A). Further cleavage with a second restriction enzyme resulted in two fragments of 2227 and 1012 bp, only the longer one being detectable by autoradiography following separation by gel electrophoresis (Fig. 8B). 3'-Labeling showed that only one of the fragment ends carried the label, i.e. the other end was blocked most likely by covalent attachment of TelN (Fig. 8B). 5'-32P-labeling indicated that both 5'-ends were accessible to the label (data not shown). These observations demonstrate that telomere resolution for the (TA)7 substrate went as far as the first cleavage reaction that yielded the covalent adduct of DNA and TelN as the important reaction intermediate. No covalently closed hairpin ends were detected by alkaline gel electrophoresis (data not shown). Obviously this finding provides a way of dissecting the transesterification reactions in telomere resolution.

Our results demonstrate that the inverted sequence repeti-
flanking formation, the pair of inverted sequence repetitions R3/L3 palindrome of telRL protein. In line with this interpretation, it is also possible that R3/L3 alters the DNA geometry of telO slightly so extended protein-DNA contacts may result in stable complex formation. telO is located in the center of tos (310 bp) and flanked by three sets of inverted repeats R1/L1 to R3/L3 (1). The apparent dissociation constant determined for tos-TelN complexes is approximately 5-fold lower than obtained for telRL-TelN complexes (Fig. 2A and Table III). How could this difference be explained?

The central problem that specific DNA-binding proteins have to solve is how to overcome the kinetic barrier of finding the cognate binding site among a large excess of nonspecific DNA. One solution to greatly facilitate this task is one-dimensional diffusion along the DNA until the target sequence is reached (34). The high unspecific DNA binding ability observed for TelN (Table III) supports the idea of binding to DNA randomly and then sliding along the double strand until the specific site has been recognized. In addition, the repeats R1/L1 and R2/L2 embedding telRL might serve as association points where the protein preferably but loosely binds and slides along the DNA until it hits telRL, where stable complex formation takes place.

**DISCUSSION**

The 56-bp telRL located within tos is the binding site for TelN, because the protein forms specific and stable complexes with telRL (Figs. 2 and 3). Because telO, the central 22-bp palindrome of telRL (Fig. 5), is no target for stable complex formation, the pair of inverted sequence repetitions R3/L3 flanking telO may provide essential recognition sequences for the protein. In line with this interpretation, it is also possible
In the cell, the preferred conformation of circular double-stranded DNA is a negatively supercoiled form I (F I). In the last replication step telomere resolution at both telLL' and telRR' results in two single linear DNA molecules with covalently closed hairpin ends telR and telL. Therefore, it is likely that structural alteration in a superhelical substrate would allow establishment of a specific DNA-TelN complex with the most suitable geometry for the cleaving-joining reaction. Such alterations might consist in cruciform extrusions, establishment of Z-conformation, or local topological changes, for instance local unwinding.

The 56-bp telRL sequence contains 2 bp flanking the central 22-bp palindromic telO on each side, which interrupt the 2-fold rotational symmetry (see Fig. 5). This asymmetry allowing differentiation between telR and telL is obviously dispensable for telomere resolution in vivo for N15 (3) and for the Borrelia linear plasmid lp17 (19) and also in vitro for N15 (Fig. 7, see below). In line with the N15 telRL, telomeres from Borrelia (chromosome and linear plasmid) also contain such slight asymmetries (see e.g. Ref. 9), whereas the telomere resolution site of the Yersinia enterocolitica phage PY54 is completely symmetric.1

Cleavage in telO occurs either in a staggered way or directly in the middle of the 22-bp sequence (Fig. 5), the latter option being rather unlikely for sterical reasons (6). Two TelN molecules are bound to telRL as demonstrated by using surface plasmon resonance (Fig. 6). Hence, one TelN molecule is supposed to bind to and occupy each arm of telRL, the region that contains both ends of the strands destined to be connected. The frictional ratio of 1.8 suggests an overall elliptic shape of TelN that may explain the extended footprint covering the complete telRL target site (Fig. 5). Telomere resolution within the 22-bp telO would not per se require TelN to cover additionally the flanking repeats R3/L3. If cleaving-joining occurs in a staggered way, each of the bound TelN molecules probably stabilizes the cleaved strands to fix the acceptor ends on the complementary strand for executing the joining reaction. However, even when we applied a telO containing plasmid substrate for the TelN-mediated telomere resolution under a variety of conditions, we never detected any intermediate. The cleaving-joining reaction proceeded fast to completion in the presence of either telRL or telO. Because the TelN concentration required for processing telO is about 50-fold higher than for telRL, we

**TABLE III**

Apparent equilibrium dissociation constants of TelN-DNA-complexes

| Substrate | \(K_{d}^{app}\) | Processability |
|-----------|---------------|---------------|
| tos       | 28            | 100%          |
| telRL     | 130           | 97%           |
| telRL A20TT21A | 290          | 0%            |
| telRL A20TT21A | 310          | 0%            |
| telIO     | 1300          | 1             |
| pBR329Δ1  | 1300          | 0             |

\(a\) TelN Y424F was used for determination of \(K_{d}^{app}\).

\(b\) Substrate processing was normalized to the activity measured for TelN wt and tos. The reaction conditions are described in the legend of Fig. 6.

\(c\) TelN wt.

\(d\) The amount of TelN applied was 100 times higher as for the tos or telRL substrate.

These repeats located in the nearest vicinity of the telomere resolution site are unique on the 46-kb N15 chromosome, where tos occupies much less than 1 bp.

Furthermore, the sets of sequence repetitions within tos may favor a locally limited structural alteration at telRL that consequently improves DNA-TelN interactions at the telomere resolution site to yield more stable complexes. If the stabilization of TelN-tos complexes we observed using a linear DNA fragment is actually caused by such conformational changes, telomere resolution in a supercoiled target may even be more favorable. Which of the two possibilities is most conceivable?

A convincing model for replication of linear genomes with covalently closed ends proposes the existence of circular head-to-head (telLL') and tail-to-tail (telRR') dimers as replication intermediates. These replicated telomeres telLL' and telRR' are completely symmetrical. The circular intermediates would then be processed by a cleaving-joining reaction, the telomere resolution, to regenerate covalently closed hairpin ends in the progeny molecules (18, 35)

Fig. 8. End labeling of TelN-generated products. A, schematic outline of the experiment. The mutant plasmid substrate carrying a central (TA) 4 instead of TATA(CG) 4TATA in the telRL (I) was linearized with NruI (II), processed with TelN (III), and treated with proteinase K. The 3239-bp fragment was isolated (IV) and \(^{32}P\)-labeled either at the 5' end using T4 polynucleotide kinase and \(^{32}P\)ATP or at the 3' end using calf thymus terminal transferase and \(^{32}P\)dATP (V). The location of the label is marked by an asterisk. Following cleavage with AatII, the products of 2227 and 1012 bp were separated by agarose gel electrophoresis (VI). B, gel electrophoresis of 3' -end-labeled fragments. The lanes are as follows: 1, DNA after cleavage with AatII (VI); 2, DNA before cleavage (V). The \(^{32}P\)-labeled fragments were stained with ethidium bromide. 1*, 2*, autoradiograph of 1 and 2. M, size marker (1-kb DNA ladder).
proposed catalytic domain box C (VXXE/LGHXXXTXHYK) are also present in TelN. The mutation Y424F in the short motifs named box A, B, and C conserved in these proteins (36) are also present in TelN. The mutation Y424F in the proposed catalytic domain box C (VXXE/LGHXXXTXHYK) is inert in telomere resolution indicating that Tyr-424 might indeed function as the key residue in catalysis. As the study of reaction intermediates is often complicated by virtue of their transient and unstable nature, our (TA), mutant will serve as a highly potent tool to address this question, because the intermediates could be isolated in sufficient amounts to determine the corresponding phosphoaminoacyl residue. The (TA), substrate will also help to precisely locate between which nucleotides both strands are cleaved.

Because the TelNY424F mutant protein is inert in processing but retains the specific binding capacity, we suspect that the protein consists of distinct domains, one of them being responsible for DNA binding. For the reasons mentioned above TelN most likely functions as a specialized tyrosine recombinase for which donor and acceptor strands reside in the same DNA molecule.

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