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Uncoupling the chemical steps of telomere resolution by ResT

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ResT is the telomere resolvase of the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease. ResT is an essential cellular function that processes replication intermediates to produce linear replicons terminated by covalently closed hairpin telomeres. ResT generates these hairpin telomeres in a reaction with mechanistic similarities to those catalyzed by type IB topoisomerases and tyrosine recombinases. We report here, that like most of the tyrosine recombinases, ResT requires inter-protomer communication, likely in an in-line synapse, to activate reaction chemistry. Unlike the tyrosine recombinases, however, we infer that the cleavage and strand transfer reactions on the two sides of the replicated telomere occur nearly simultaneously. Nonetheless, the chemical steps of the forward and reverse reactions performed by ResT can occur in a non-concerted fashion (i.e. events on the two sides of the replicated telomere can occur independently). We propose that uncoupling of reaction completion on the two sides of the substrate is facilitated by an early commitment to hairpin formation that is imposed by the pre-cleavage action of the hairpin binding module of the ResT active site.

Spirochetes of the genus *Borrelia* are important human pathogens (see (1,2)) that cause Lyme disease (3,4) and relapsing fever (5). The *Borrelia* species possess intriguing genomes in that they are highly segmented with most of their replicons being linear DNAs terminated by covalently closed DNA hairpins, referred to as telomeres (see (6)).

The strategy employed by *B. burgdorferi* to propagate its linear replicons has been described recently (see (7,8)). Replication initiates from an internal origin and proceeds bidirectionally (9). Passage of the replication forks through both of the hairpin telomeres would produce an inverted repeat circular dimer replication intermediate. The resulting replicated telomeres are processed by DNA breakage and reunion events (telomere resolution) that liberate the linear daughters from the presumed circular dimer intermediate (10). The enzymatic activity that performs telomere resolution is an essential cellular function provided by ResT, the product of the BBB03 locus of cp26 (11,12). A similar replication strategy has been demonstrated for the N15 bacteriophage, the best characterized example of the bacteriophages that possess linear lysogenic genomes terminated by hairpin telomeres (13-15).

Telomere resolution by ResT proceeds by a two-step transesterification involving an active site tyrosine (Y335) that proceeds with the same polarity (a DNA-3'phosphotyrosyl-enzyme intermediate) as that of the type IB topoisomerases and the site-specific recombinase class referred to as tyrosine recombinases (11,16). Nucleophilic attack by the active site tyrosine, on

phosphodiester bonds separated by six bp on the opposite DNA strands in the replicated telomere, produces a transient covalent 3'-phosphotyrosyl-ResT intermediate. Subsequent nucleophilic attack of the phosphotyrosyl linkage by the 5' hydroxyl group of the opposite DNA strand displaces the resolvase and results in the formation of covalently closed hairpin telomeres (Fig. 1A, top panel). ResT can also run the reverse reaction (Fig. 1A, bottom panel) in which hairpin telomeres are fused together, an activity believed to generate telomere exchanges and gene duplications among the linear replicons of *B. burgdorferi* (17).

The composite active site of ResT has catalytic residues similar to those described for type IB topoisomerases or tyrosine recombinases (16) but also incorporates a hairpin binding module similar to that found in cut-and-paste transposases of the Tn5 and Tn10 elements, which use a DNA hairpin intermediate (18-20) . The action of the hairpin binding module to distort the DNA between the cleavage sites is required to activate DNA cleavage (21) and the two active site components explain the unique activity of telomere resolvases. To gain a further understanding of the mechanistic properties of this unique class of enzymes we performed experiments to ask whether there was a requirement for concerted DNA cleavage and strand transfer for the telomere resolvase ResT.

We report here that the action of ResT in both chemical steps of the forward and reverse reactions can occur in a non-concerted fashion. That is, for either cleavage or strand transfer, the events occurring on opposite sides of the axis of symmetry can be uncoupled. Nonetheless, our data show that inter-protomer communication across the symmetry axis is required to initiate reaction chemistry, which likely occurs in the context of an 'in-line' synapse of ResT in both the forward and reverse reactions. These results are discussed and contrasted with the reaction details of the mechanistically-related topoisomerases and conservative site-specific recombinases.

Materials and Methods

Substrate DNAs - For Fig.2, the asymmetric wt-nick/OPN[1] rtel was assembled with oligonucleotides OGCB2 and 3, comprising the 70 bp side of the rtel, these oligos are reported in (10). OGCB 179 (5'-gatcCACTCTATACT AATAAAAAATTA*T-3') and OGCB18 (5'-atatATAATTTTTATTAGTATAGAGTG-3') make up the 25 bp side of the rtel; the asterisk marks the position of the 5' bridging OPN modification of the cleavage site. OGBC3 was kinased and annealed to OGCB2 while OGCB179 and 18 were annealed without kinase treatment. The annealed halfsites were mixed and ligated at 8^{o}C overnight with T4 DNA ligase (New England Biolabs). The 105 bp wt-nick/OPN rtel was purified away from the 70 and 140 bp rtels also assembled in the ligation by the crush and soak method from a 5% PAGE 1XTAE native gel. The wt-nick/wt rtel control was assembled by the same method except OGCB179 was substituted by OGCB17 which has the same sequence but lacks the OPN modification of the cleavage site.

For Fig. 3 the asymmetric wt/wt and OPS/wt rtels were assembled with OGCB 137 (5'-CTAGGGGGGGAATCTA-3'), OGCB 138 (5'-TACTAATAAAAAATTA*TA TATATAAT-3'), OGCB 139 (5'-TTTTTATTAG TATAGATT-3'), OGCB 140 (5'-GATCAATCTA TACTAATA-3'), OGCB 141 (5'-AAAAATTAT ATATATAATTTTTTATT-3'), OGCB 142 (5'-AGTATAGATTCCCCCC-3'). For the OPS modification OGCB 145 was used which is OGCB138 with a 5' bridging OPS modification at the cleavage site, marked by the asterisk in the OGCB 138 sequence. OGCB 138, 139, 141, 142 and 145 were kinased with Polynucleotide kinase (New England Biolabs) in T4 DNA ligase buffer. The kinase was heat inactivated at 65^{o}C for 45 minutes. Then the following pairings of oligonucleotides were combined in buffer supplemented with 50 mM NaCl and annealed by heating at 95^{o}C for 3 minutes followed by slow cooling to room temperature: OGCB 137/142, OGCB 138/141, OGCB 145/141, and OGCB 139/140. Then OGCB 137/142, 138/141 and 139/140 and OGCB 137/142, 145/141 and 139/140 were pooled, supplemented with ATP to 1 mM and ligated at 16^{o}C for 2 hours to assemble the wt/wt and OPS/wt rtels, respectively. The rtels were gel purified as previously described (17).

For Fig.4 the OPS, OPN and mock telomere hairpins were made by snapback cooling of rtels assembled with OGCB (OPS) 116 (5'-gatcCACTCTATACTAATAAAAAATTA*TATA T-3') and OGCB 117 (5'-ATAATTTTTTA

TTAGTATAGAGTG-3') for the OPS hp and OGCB 179 and OGCB 18 for the OPN hp and OGCB 8 and 9 for the mock telomere hairpin. The asterisk marks the position of the cleavage site and the 5' bridging OPS modification. Rtel assembly and snapback hairpin formation was performed as described in (10,17). For Fig. 5 the 35 bp unmodified hp was obtained by snapback cooling of an rtel assembled with OGCB 4 and 5 (10).

For Fig. 6, the half-sites were assembled by annealing OGCB 17 with OGCB 117 for the (-2bp) half-site; OGCB 18 and OGCB 116 for the (+2bp) half-site; OGCB 191 (5'-gatcCAC TCTATACTAATAAAAAATTA*TAT-3') and OGCB 192 (5'-ATATAATTTTTTATTAGTAT AGAGTG-3') for the 0 bp half-site. The asterisk marks the position of the cleavage site. The control blunt-ended site was a 30 bp Tn5 outside end assembled by annealing Tn5-NTS-30 (5'-CTGTCTCTTATACACATCTTGAGTGAGTGA-3') and Tn5-TS-30 (5'-TCACTCACTCAAGA TG TGTATAAGAGACAG-3')

Proteins - Untagged wild-type recombinant ResT was overexpressed and purified from E. coli as previously described (11).

In Vitro Reaction Conditions - Unless otherwise stated in the figure legends the reactions were incubated in 25 mM Tris-HCl (pH 8.5), 1 mM EDTA (pH 8.0), 100 mg.ml BSA and 100 mM NaCl at 30^{o}C.

Gel Electrophoresis and Documentation - Native PAGE gels (15 cm x 15 cm) were of the indicated percentages for acrylamide (29:1 acrylamide to bis-acrylamide) and were run at room temperature in 1xTAE at 10 V/cm for 70 min. SDS-PAGE gels were run as per the native gels, but supplemented in the gel and running buffer with 0.1% SDS. Denaturing PAGE gels (40 cm x 20 cm) were urea/TBE gels run at a temperature of 50^{o}C in 1xTBE at 45V/cm for 50 min. All radioactive gel bands were quantified and/or visualized using a Phosphorimager from Molecular Dynamics.

RESULTS

Tests for concerted cleavage and strand transfer in the forward reaction – For the purposes of this study we define concerted reaction chemistry as an obligatory requirement for the coordinated action of the two active sites for the cleavage or strand transfer steps occurring on the two sides of the replicated telomere. We first determined whether the two DNA cleavage events in the ResT forward reaction, which occur on opposite sides of the axis of symmetry, must occur in concert. An oligonucleotide comprising an asymmetric replicated telomere (rtel) was employed as a substrate to allow cleavage events on either side of the axis to be distinguished from each other. On the left side of the axis of symmetry the substrate (wt-nick/OPN rtel) contained an unmodified cleavage site immediately followed by a nick (Fig. 2A). Cleavage at this site results in the liberation of a single nucleotide adjacent to the cut site, which blocks resealing of the cleavage site by reversal of the ResT reaction and traps cleaved product. On the right side of the axis the substrate contained an un-cleavable OPN modification (see Fig. 1B). If the DNA cleavage events must occur in concert, then blocking cleavage on one strand (with the OPN modification) would also block cleavage of the other strand, and the wt-nick/OPN rtel would remain unreacted (Fig. 2A, scenario i). If the cleavage events need not be concerted, a covalent-protein DNA complex (CPD) would be trapped after cleavage on the left side of the axis because of the adjacent nick (Fig. 2A, scenario ii). The data documented in Fig. 2B revealed that reaction with a wt-nick/OPN rtel yielded nicked rtel CPD's corresponding to 63% of the input substrate (lane 1), while incubation of ResT with the wt-nick/wt rtel substrate produced 61% reaction of the input substrate (lane 3). Treatment of the observed products with pronase confirmed their assignment as CPDs (lane 2 and 4). This indicated that the DNA cleavage events need not occur in concert; blocking cleavage on one strand did not block cleavage of the opposing strand, thus supporting scenario ii. Reactions with the control wt-nick/wt rtel substrate confirmed that the OPN modification was indeed blocking cleavage, since the reaction products obtained from bottom strand cleavage of the control were absent from the reaction with the wt-nick/OPN rtel substrate. The comparable reaction levels with the two substrates also indicated the non-concerted cleavage observed with the wt-nick/OPN rtel was not the result of a rare side reaction. A timecourse with the wt-nick/wt rtel also revealed that the intact strand is cleaved at approximately twice the rate of the nicked strand (data not shown); therefore, the observed level of the 95 bp CPD band (lane 1) is

likely an underestimate of the non-concerted reaction product. Similarly, in experiments in which one scissile phosphate was pre-cleaved in an rtel, cleavage of the other strand was not blocked in studies with the bacteriophage telomere resolvases TelN and TelK (22).

Next, we asked whether the pair of DNA strand transfer events catalyzed by ResT must occur in concert with each other during the forward reaction. To test this, an oligonucleotide substrate comprised of an asymmetric replicated telomere was employed to allow reaction products from either side of the axis to be distinguished from each other. The OPS/wt rtel used (Fig. 3A) contained an OPS modification of one cleavage site while the cleavage site on the other side of the axis was left unmodified. The OPS modification supports cleavage but not subsequent strand transfer (see Fig. 1B). If hairpins must be formed in concert, then the OPS substitution on one side of the axis would block hairpin formation on both sides and only half-site CPDs would be recovered (Fig. 3A, scenario i). If the strand transfer reactions need not occur in a concerted manner, then a CPD and a hp would result (Fig. 3A, scenario ii). The data from the non-denaturing (for DNA) SDS polyacrylamide gel shown in Fig. 3B revealed that both a half-site CPD and a hairpin telomere were produced in the reaction with the OPS/wt rtel substrate (lane 3), indicating that the strand transfer events that produce the hairpins need not proceed in concert. Substrate utilization of the OPS/wt rtel was 73% compared to 74% for the wild type telomere. Denaturing gel analysis (Fig. 3C) also showed that the yield of the single hp resulting from reaction with the OPS/wt rtel was undiminished relative to reaction with the wt/wt rtel (compare lanes 5 & 6). It is noteworthy that blocking hairpin formation on one side of the site did not induce ResT to reseal the parental strand competent for ligation, to regenerate a nicked rtel (see the discussion for further elaboration of this point). In studies with the bacteriophage telomere resolvases, TelN and TelK, nicks in certain positions in the rtel substrate were also found to support non-concerted strand transfer events. In contrast, nicks anywhere between the cleavage sites blocked formation of both hairpins with the phage resolvases (22). Our wt-nick/wt rtel control for Fig. 2 did not show a defect for hairpin formation on the side of the site that still had the appropriate nucleophile (Fig. 2C and data not shown).

Tests for concerted cleavage and strand transfer in the reverse reaction - We next turned our attention to the issue of whether the reaction chemistry performed by ResT in the reverse reaction (telomere fusion) must be concerted. For the cleavage step of the reverse reaction an assay was devised in which a labeled OPS hp was paired for reaction with unlabeled, non-cleavable OPN hp (see Fig. 4A). The result expected from this pairing depends upon two parameters: whether DNA cleavage occurs in the context of a synapse of hairpin telomeres or not, as well as whether the cleavage events in such a putative synapse are obligatorily concerted. By definition, concerted cleavage events can only occur in a synapse, while non-concerted cleavage can be the result of cleavage of individual hp's or an uncoupling of the cleavage events in the context of a synapse.

To simultaneously probe both these issues we added increasing amounts of unlabeled OPN hp or mock hp telomere into cleavage reactions with the OPS hp reporter, using a concentration of OPS hairpin telomere that resulted in suboptimal cleavage in an unsupplemented reaction. If the DNA cleavage in the reverse reaction must be concerted (scheme i) or occurs on individual hp telomeres (scheme iia) then addition of increasing amounts of unlabeled OPN hp would inhibit cleavage of the labeled OPS hp reporter by communication to the second active site in the former case, or by competing for ResT, in the latter. In contrast, if the DNA cleavage events in the reverse reaction need not occur in concert, but ResT is activated for DNA cleavage in the context of a synaptic hp complex, then addition of increasing amounts of unlabeled, non-cleavable OPN hp should stimulate CPD formation on the labeled OPS hp reporter by increasing the concentration of synaptic complexes (scheme iib).

As shown in Fig. 4B addition of OPN hp to reactions with the OPS hp reporter stimulated CPD formation in a concentration-dependent manner at either the optimal reverse reaction temperature of 8^{o}C or the standard 30^{o}C resolution temperature. Addition of the same amounts of mock hp telomere had no effect on cleavage of the OPS hp, controlling for stimulation from trivial effects on protein solubility/activity derived from higher concentrations of DNA in the assay. These

results indicate that hairpin telomere cleavage need not be concerted in the reverse reaction, but is activated by association of hairpin telomeres.

The strand transfer step of the reverse reaction was also examined for concerted versus non-concerted events. For the strand transfer step an assay was designed in which a labeled OPS hp was paired for reaction with an unlabeled, unmodified hp (Fig. 5A). Upon cleavage of the OPS hp, the resulting 5'-SH group is incompetent for strand transfer. Therefore, joining on one of the two strands in the reverse reaction of an OPS hp coupled with an unmodified hp would be blocked. If the strand transfer events in telomere fusion must be concerted, then the OPS hp should be unable to participate in the reverse strand transfer reaction and should be arrested at the CPD intermediate (scheme i). On the other hand, if the strand transfer events can be uncoupled from each other then a nicked rtel in a covalent complex with ResT would result (scheme ii). Fig. 5B shows that after protease digestion to remove covalently bound ResT, a wild type yield of rtel (joined on one strand) was observed for the reaction between a 25 bp OPS hp reporter and a 35 bp unmodified hp (compare lanes 2 vs 4). This indicates that the strand transfer events for the reverse reaction need not occur in concert.

Communication between half-sites is required for ResT-mediated DNA cleavage - Experiments with a half-site carried on a plasmid indicated that a single half-site was insufficient for cleavage (data not shown), suggesting a necessity for communication of ResT molecules bound on both sides of the symmetry axis to activate reaction chemistry in the forward reaction. Similarly, in the reverse reaction, stimulation of hairpin cleavage by increasing concentrations of substrate (Fig. 4) suggested that association of ResT-bound hp telomeres into a synapse facilitates their cleavage through ResT communication across the synapse. To test for communication across a specific 'in-line', head-to-head synapse relevant to both the forward and reverse reactions, we examined ResT cleavage of blunt-ended half-site substrates under conditions favoring or precluding the formation of an in-line synapse.

The ability of ResT to cleave half-sites allowed us to manipulate the length of the half-sites to influence the ability of in-line synapsis to occur (Fig. 6A). Blunt-ended half-sites that are the same chain length as hairpin telomeres are optimal cleavage substrates; extending or shortening the half-site by 2 bp results in 4-fold and 84-fold reduction in the rate of DNA cleavage, respectively (Fig. 6 and data not shown).

If the diminished ability of ResT to cleave a shortened (-2 bp) telomere was due to a failure to synpase half sites head-to-head, then the cleavage defect for these half sites should be rescued by addition of partner sites that provide the missing base pairs needed to form a properly aligned synapse. Fig. 6 presents the results of such experiments for the profoundly defective (-2 bp) half-site. This half-site showed only trace levels of cleavage (Panel B1). Supplementing with a control double stranded oligonucleotide of non-telomeric sequence did not result in stimulation of cleavage (Panel B2). Similarly, the presence of additional (-2 bp) half-site did not give significant stimulation of the reaction (Panel B3). In contrast, the reaction was partially rescued by addition of a half-site that terminates at the axis of symmetry (0 bp, Panel B4) and fully rescued to give 60% cleavage of the input substrate by addition of a (+2 bp) half-site (Panel B5). The simplest way to interpret the rescue afforded by the (+2 bp) half-site, is for its addition to provide the base pairs missing in the (-2 bp) reporter, allowing re-establishment of proper communication across a head-to-head, in-line synapse. The sum of our data in this section indicate that ResT is not active as a monomer on half-sites and that communication between half-sites occupied by ResT, be they the two halves of a replicated telomere or two hairpin telomeres in a synapse, is required to activate ResT for DNA cleavage.

DISCUSSION

The ResT connection to topoisomerases and site-specific recombinases - Topoisomerases, conservative site-specific recombinases and telomere resolvases share a common chemical mechanism of DNA phosphoryl transfer proceeding via a two-step transesterification. The first event, which involves covalent linkage of an enzyme sidechain (tyrosine or serine) stores the DNA bond energy of the cleaved strand in a covalent enzyme-DNA intermediate. The liberated hydroxyl group of the cleaved DNA initiates the second transesterification on the enzyme-DNA

complex, to achieve strand transfer or rejoining (see (23) for a general discussion of chemical mechanisms). Though the site-specific recombinases and topoisomerases employ this common chemical mechanism, the nature of their products vary because of the number of the DNA strands cleaved, and the identity of the strands they are joined to. Topoisomerases cleave one or two DNA strands that are rejoined after strand passage to change levels of DNA supercoiling or catenation (24-26). The site-specific recombinases cleave four DNA strands and rejoin them to new partners to make recombinant products. The complexity of the telomere resolution reaction performed by ResT lies midway in this spectrum. Telomere resolution can be considered as a stripped down recombination reaction in which strand transfer occurs within the replicated telomere rather than to a recombination partner (13,27-29).

These differences in the number and identity of DNA strands cleaved and rejoined are accompanied by differences in the requirements for coordination between active sites and in the timing of the chemistry on the two strands of a target site. The type II topoisomerases that cleave and rejoin two DNA strands appear to have active sites that act independently with both the cleavage and strand transfer events occurring in a non-concerted manner (30-32). Within the site-specific recombinases two different approaches to the coordination and timing of the DNA cleavage and strand transfer events exist between the serine and tyrosine recombinases. The serine recombinases bring two recombination sites together, cleave four DNA strands and after a synaptic rearrangement equivalent to a right-handed 180^{o} rotation, execute four concerted strand transfer events to generate the recombinant product (33). In contrast, the tyrosine recombinases bring together two recombination sites and execute pairwise DNA cleavage and strand transfer reactions of the equivalent strand in each duplex to form a Holliday junction intermediate. Recombinant products are then produced by an isomerization of the Holliday junction that activates the remaining pair of active sites for cleavage and strand transfer of the other pair of DNA strands (34).

For the serine recombinases the reactions performed by a dimer bound to a crossover site are largely simultaneous and concerted (33,35), whereas, for the tyrosine recombinases a strict temporal order at individual half sites is enforced; only one active site in a dimer bound to a recombination site can be active at a time (28,36). The findings in this study indicate that ResT falls midway in this spectrum. Like the site-specific recombinases and type II topoisomerases, ResT appears to require inter-protomer communication across the symmetry axis to initiate reaction chemistry, which occurs in the context of an 'in-line' synapse of ResT. Additionally, while we found the cleavage and strand transfer reactions on both sides of the symmetry axis occur nearly simultaneously, we also found that the chemical steps of the forward and reverse reactions can occur in a non-concerted fashion. That is, for either cleavage or strand transfer, the events occurring on opposite sides of the axis of symmetry can be uncoupled (see below).

Mechanistic Implications - Although ResT uses reaction chemistry analogous to that of the tyrosine recombinases and shares some active site features with these enzymes, it is apparent that differences in the reaction preclude the use of a similar strict temporal order (cleavage followed by strand transfer) at each individual half site (36,37). This is because hairpin formation on one side of the axis is dependent upon generation of an attacking nucleophile on the opposite side of the axis, produced by resolvase-mediated DNA cleavage. Nonetheless, a mechanism involving a strict temporal order with oscillation of active site activity from one side of the axis to the other ($\text{cleavage}_{\mathbf{A}} \Rightarrow \text{cleavage}_{\mathbf{B}} \Rightarrow \text{strand transfer}_{\mathbf{A}} \Rightarrow \text{strand transfer}_{\mathbf{B}}$) is theoretically possible. Several lines of evidence argue that ResT does not employ this type of ordered step-wise mechanism: 1) Very little singly nicked or singly hairpinned species are detectable in the course of unperturbed reactions (data not shown). 2) Blocking cleavage on one strand with the OPN modification does not block cleavage on the other strand (Fig. 2) as it does for a tyrosine recombinase (38). 3) The forward trajectory of the reaction towards completion (hairpin formation) on one side of the axis is maintained even when hairpin formation is blocked on the other side of the replicated telomere (Fig. 3). If the reaction were step-wise, stalling of ResT at a hairpinning step it could not perform would reseal the one parental strand competent for ligation, regenerating a nicked rtel.

This would be a safer outcome for the cell than the single hp actually formed at the expense of the double-strand break made on the other side of the site. The tyrosine recombinases, which use a step-wise mechanism, usually regenerate substrate when they cannot complete a full reaction cycle (38-40).

The surprising combination of simultaneous action on both sides of an rtel with the freedom for uncoupling completion of the reaction on the two sides of the site can be rationalized in terms of substrate commitment. We propose that an early, pre-cleavage commitment to hairpin formation in the forward reaction explains this unusual mode of action. ResT has a composite active site; besides employing topo IB or tyrosine recombinase-like catalytic residues (16), the ResT active site also contains a hairpin binding module that acts before DNA cleavage to distort the DNA between the cleavage sites to activate cleavage and to aid in hairpin formation (21). Heteroduplexing the DNA between the cleavage sites rescues the defect of mutants in this module (21). The commitment to hairpin formation, revealed by the experiment in Fig. 3, and the low yield of CPD in normal reactions, argue that the 5' OH used in hairpin formation is positioned by the hairpin binding module, to facilitate very rapid transesterification to the 3'-phosphotyrosyl complex on the opposite strand. This is reminiscent of the activity of the tyrosine recombinases, Flp and λ integrase, on substrates in which the entire sequence between the cleavage sites has been heteroduplexed (27,28). Such heteroduplexed substrates appear to be processed by simultaneous cleavage of the two strands, and because intermolecular strand transfer and simple strand resealing are blocked by the sequence heterology, DNA hairpins are produced instead. So for ResT, mixing a Tn5 transposase-like hairpin binding module with a tyrosine recombinase-like active site, produces these unique reaction features, previously only observed for tyrosine recombinases with substrates carrying completely heteroduplexed DNA between the cleavage sites.

Biological Implications - It should be noted that the requirement for ResT communication across an in-line synapse to promote DNA cleavage (Fig. 6) allows opportunities for the regulation of its activity *in vivo*. This is especially pertinent with respect to the reverse reaction. We have discovered recently that ResT interacts with its hairpin product with an affinity nearly equal to that of its affinity for the rtel substrate and that it can catalyze hp cleavage and hp telomere fusion (17). If ResT could cleave hp telomeres outside the relatively safe context of a synapse, frequent double-strand break formation, loss of genetic material, and reduction in virulence or bacterial cell viability would result. It will be especially interesting, in future studies, to determine if the regulation of ResT activity afforded by the requirements for 'in-line' communication operates by allosteric domain swapping (used by the bacterial tyrosine recombinases that cleave in *cis*) or by contribution of the active site tyrosine in *trans* (as for *S. cerevisiae* Flp) (41-46).

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FOOTNOTES

[1]The abbreviations used are: OPN, 5' bridging phosphoramidate; OPS 5' bridging phosphorothiolate; CPD, covalent protein-DNA complex; rtel, replicated telomere; hp, hairpin

FIGURE LEGENDS

Fig. 1. The forward and reverse reactions performed by ResT.
A) In telomere resolution by ResT, a replicated telomere is resolved into two covalently closed hairpin ends through a two step transesterification (11,16). Step one is a nucleophilic attack, by the active site tyrosine (Y335) of ResT on the scissile phosphates positioned 6 bp apart on opposite strands surrounding the symmetry axis (thick central line). Nucleophilic attack of the resulting 3'-phosphotyrosyl-ResT complexes by the 5' hydroxyls on the opposing strands of the DNA produces two covalently closed DNA hairpin telomeres. Reaction reversal (telomere fusion) is the exact chemical reversal of telomere resolution in which two hairpin telomeres are fused (17). **B)** Structures of the cleavage site modifications used in this study. The 5' bridging oxygen, which is the leaving group for the DNA cleavage step in the first transesterification, and the attacking nucleophile in the second transesterification, is changed to either a nitrogen or a sulphur to yield the OPN or OPS substituted cleavage sites, respectively. The 5' bridging nitrogen constitutes a poor leaving group and therefore the OPN site is not cleaved(47,48). The 5' bridging sulphur is a good leaving group but a poor nucleophile and therefore, the OPS site is cleaved but does not support subsequent strand transfer; instead ResT is trapped as a covalent protein-DNA complex (CPD) (48-50).

Fig. 2. Analysis of the telomere resolution cleavage step.
A) Experimental design to assess whether the DNA cleavage events on both sides of the axis of symmetry are strictly concerted. *=5' ^{32}P end label; •= cleavage site; hp= hairpin telomere; rtel= replicated telomere; CPD= covalent protein-DNA complex; OPN= 5'-bridging phosphoramidate modification at the cleavage site. The wt-nick/OPN rtel only has one cleavage site competent for cleavage as the OPN modification blocks cleavage of the bottom strand. i) If the reaction is strictly concerted, preventing DNA cleavage at the OPN modified site would also block cleavage of the top strand and no products would result from incubation with ResT. ii) If the DNA cleavage events need not be concerted then the top strand would be cleaved, liberating a single nucleotide between the cleavage site and the pre-existing nick. Diffusion of this nucleotide would block resealing and a 95 bp CPD would accumulate.
B) The control wt-nick/wt rtel has two competent cleavage sites. Cleavage of the top strand proximal to the nick liberates the single nucleotide adjacent to the cleavage site by diffusion. This inhibits top strand resealing, yielding the 95 bp CPD, and inhibits hairpin formation on the right-hand side of the rtel, yielding the 25 bp CPD from bottom strand cleavage events. Hairpin formation on the left-hand side of the rtel is mostly unaffected, though a population of molecules cleaved on both strands but not yet hairpinned on the left-hand side of the rtel yields the 70 bp CPD.
C) Autoradiograph of the SDS-PAGE (8%) analysis of telomere resolution on the asymmetric replicated telomere substrates depicted in Panel A. Reactions were incubated at 30^{o}C for 10 min with 4nM 5' labeled replicated telomere and 148 nM ResT. Reaction aliquots were stopped by addition of SDS-containing loading dye (0.1% final SDS concentration). Pronase treatment was with 500 Units in the loading dye at room temperature. Schematics of the reaction products are noted to the right of the gel.

Fig. 3. Analysis of the telomere resolution strand transfer step.
A) Experimental design to assess whether the strand transfer events involved in hairpin formation on both sides of the axis of symmetry are strictly concerted. i) If concerted, the arrest of strand transfer on one side of the axis by the presence of an OPS site would also block strand transfer on the other side of the axis, with no hairpin products being formed. ii) If the reaction need not be concerted, arrest of strand transfer on one side of the axis would not affect strand transfer on the other side, and hairpin product formation would occur with the right half of the substrate. OPS/wt rtel is an asymmetric replicated telomere with an OPS modification introduced at one cleavage site in order to block one strand transfer event. See Fig. 2 legends for other abbreviations and symbols. **B)** Autoradiograph of native gel (8% SDS-PAGE) analysis of telomere resolution on a wild type replicated telomere and a telomere with an OPS modification of one cleavage site (substrate depicted in Panel A). Reactions were incubated at 30^{o}C for 30 min with 4 nM 5' labeled asymmetric telomere and 74 nM ResT. Reaction aliquots loaded onto the SDS-PAGE gel were stopped by addition of SDS-containing load dye. The gel was run in 1xTAE and 0.1%SDS. S= substrate; P= product. Pronase treatment was with 500 Units in the loading dye at room temperature. **C)** Autoradiograph of denaturing gel (8% TBE/urea) analysis of reactions described in Panel B. Reaction aliquots loaded onto the 8% TBE/urea gel were terminated by addition of formamide loading dye and heating to 95^{o}C for 5 min prior to loading.

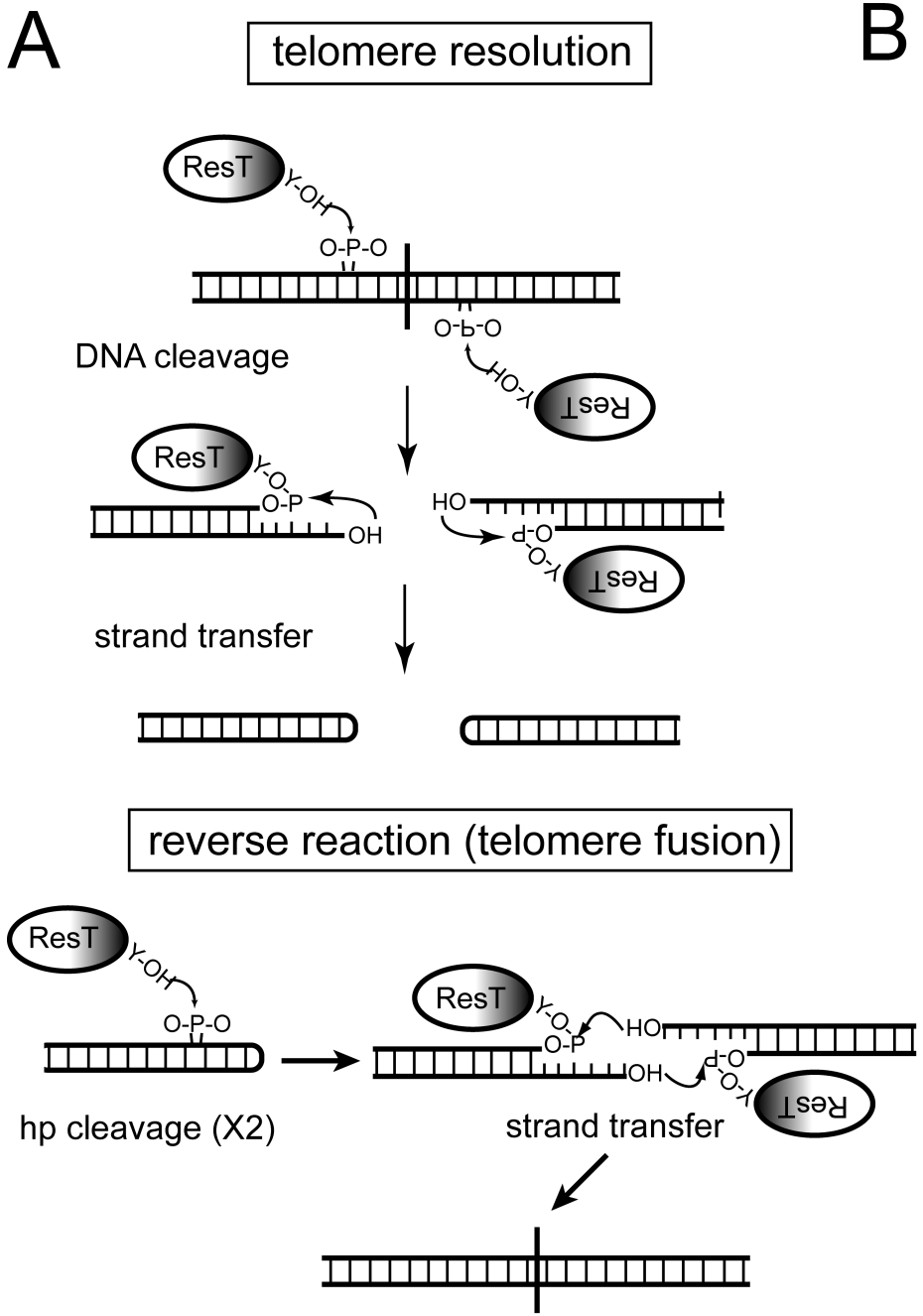
Fig. 4. Analysis of the telomere fusion cleavage step.
A) Experimental design to assess whether the DNA cleavage events on both sides of the axis of symmetry are strictly concerted in the reverse reaction. A requirement for concerted DNA cleavage of hairpin telomeres (hp) presupposes their synapsis for cleavage to occur. i) Addition of increasing amounts of non-cleavable OPN hp to an OPS hp reporter would inhibit cleavage and reduce CPD levels if the reaction were strictly concerted. ii) If cleavage of hairpin telomeres need not be concerted, then the addition of increasing amounts of non-cleavable OPN hp to reactions with an OPS hp reporter would depend upon whether cleavage occurred on individuals hp's (a) or in a synpase of two hp's (b). See Fig. 2 legends for other abbreviations and symbols. **B)** Autoradiograph of SDS-PAGE (8%) analysis of cleavage of an OPS hp in the presence of increasing amounts of a non-cleavable OPN hp. Reactions were incubated at 8^{o}C for 16 h with 148 nM ResT, 8nM 5' labeled 25 bp OPS hp and with 0, 30, 60 or 120 nM unlabeled OPN hp. 30^{o}C reactions were incubated for 10 min with 148 nM ResT, 8nM 5' labeled 25 bp OPS hp and 0, 15, 30, or 60 nM unlabeled OPN hp or with 15, 30 or 60 nM unlabeled mock telomere hp (a hairpin with no sequence resemblance to *Borrelia* telomeres).

Fig. 5. Analysis of the telomere fusion strand transfer step.
A) Experimental design to assess whether the strand transfer events on both sides of the axis of symmetry are strictly concerted for hairpin telomere fusion. i) If strictly concerted, the arrest of strand transfer on one side of the axis would also block strand transfer on the other side of the axis, with no hairpin fusion product being formed. ii) If the reaction need not be concerted, arrest of strand transfer on one side of the axis would not affect strand transfer on the other side, and hairpin fusion would occur to generate a 60 bp replicated telomere with a single nick and a covalently attached ResT on one side. **B)** Autoradiograph of native PAGE (8%) analysis of reverse telomere resolution between OPS hp and an unmodified hairpin telomere. Reactions were incubated at 8^{o}C for 16 h with 15 nM 5' labeled 25 bp OPS hp, an 8-fold molar excess of unlabeled, unmodified 35 bp hairpin telomere (where indicated) and 148 nM ResT. Reaction aliquots were stopped and treated with pronase as noted previously for Figs. 2 & 3.

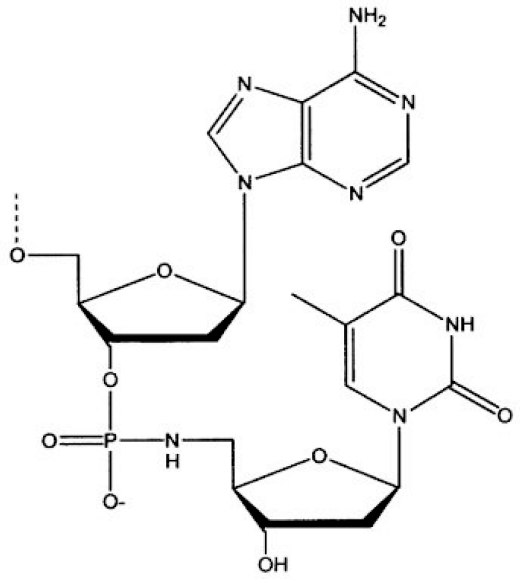
Fig. 6. Communication between half-sites is required for ResT mediated DNA cleavage.
A) Experimental design to detect 'in-line' synapsis and cleavage of half-sites. A 5' labeled, poorly cleaved half-site that includes the cleavage site but terminates 2 bp short of being a full half-site (-2 bp)*, is independently paired with a 4-fold molar excess of different unlabeled half-sites: another (-2 bp) half-site; a full half-site (0 bp); a half-site extending 2 bp beyond the symmetry axis (+2 bp). ResT-mediated DNA cleavage is assayed by measuring the formation of CPD's on the labeled (-2 bp)* half-site. CPD's

are trapped because DNA cleavage is rapidly followed by diffusion of the single nucleotide adjacent to the cleavage site thereby blocking strand transfer or resealing of the nick. 'In-line' communication between half-sites is detected by rescue of the cleavage defect on the (-2 bp) half-site. **B)** Autoradiographs of SDS-PAGE (10%) analysis of the kinetics of cleavage of the (-2 bp) half-site reporter when supplemented with various partner half-sites as indicated by the cartoons in each gel. Panels 1 and 2 are controls where either no partner was added (1) or a partner of non-telomeric sequence was added (2). Reactions were incubated at 30^{o}C with 3.75 nM 5' end-labeled (-2 bp) half-site, 15 nM unlabeled half-site partner and 185 nM ResT. Aliquots were removed at the indicated timepoints into gel loading buffer supplemented with SDS to 0.1%. **C)** Graphical representation of the data in part B).

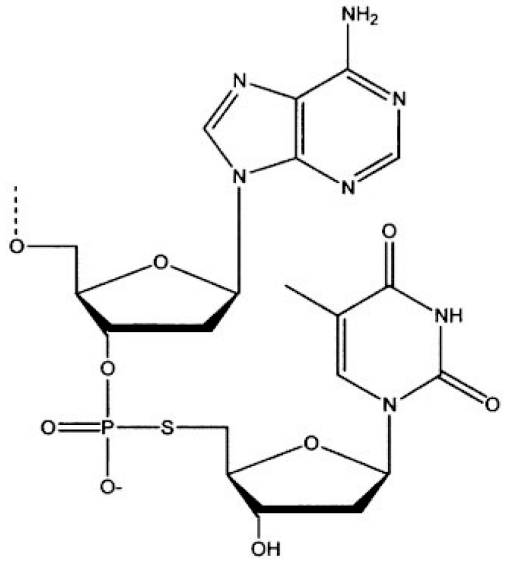
A



B



5'- bridging phosphoramidate
(OPN) modification
NON-CLEAVABLE



5'-bridging phosphorothiolate
(OPS) modification
NON-LIGATABLE

Figure 1

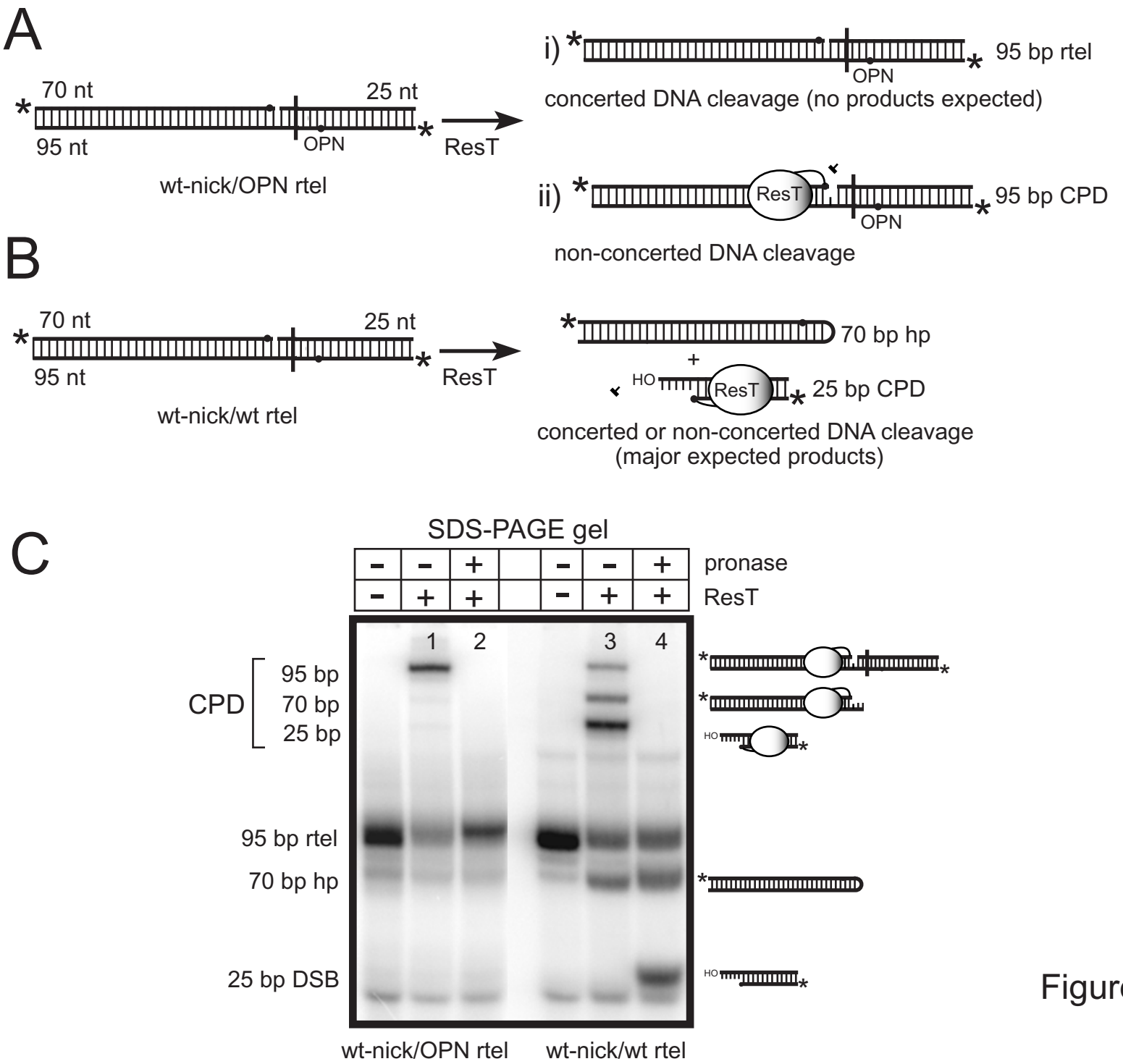

A
70 nt
25 nt
95 nt
OPN
ResT
wt-nick/OPN rtel
i)
95 bp rtel
concerted DNA cleavage (no products expected)
ii)
ResT
95 bp CPD
non-concerted DNA cleavage
B
70 nt
25 nt
95 nt
ResT
wt-nick/wt rtel
70 bp hp
HO
25 bp CPD
concerted or non-concerted DNA cleavage
(major expected products)
C
SDS-PAGE gel
pronase
ResT
1
2
3
4
CPD
95 bp
70 bp
25 bp
95 bp rtel
70 bp hp
25 bp DSB
wt-nick/OPN rtel
wt-nick/wt rtel

Figure 2

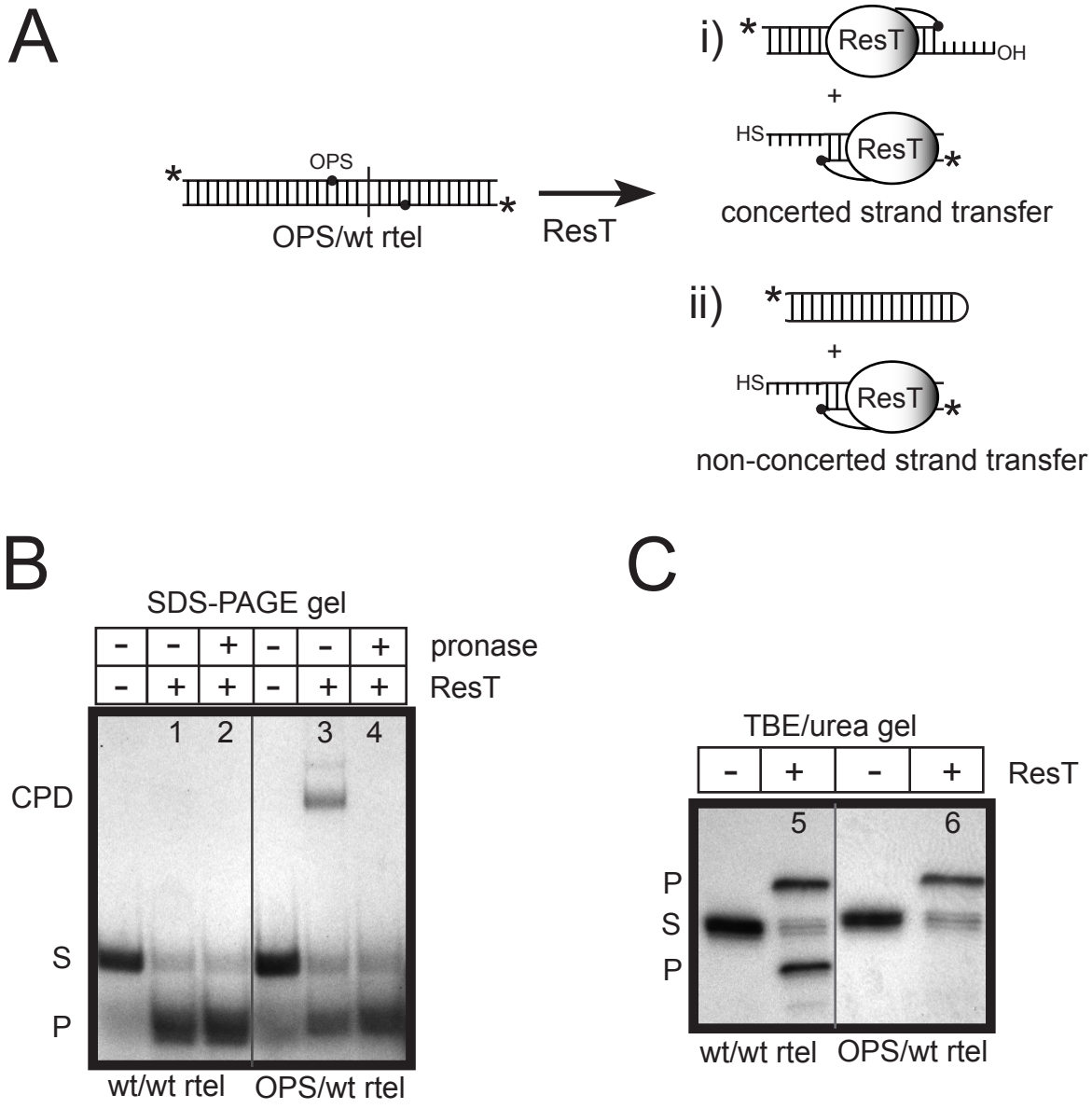

A
OPS
OPS/wt rtel
ResT
i)
ResT
OH
HS
ResT
concerted strand transfer
ii)
HS
ResT
non-concerted strand transfer
B
SDS-PAGE gel
pronase
ResT
1 2 3 4
CPD
S
P
wt/wt rtel
OPS/wt rtel
C
TBE/urea gel
ResT
5 6
P
S
P
wt/wt rtel
OPS/wt rtel

Figure 3

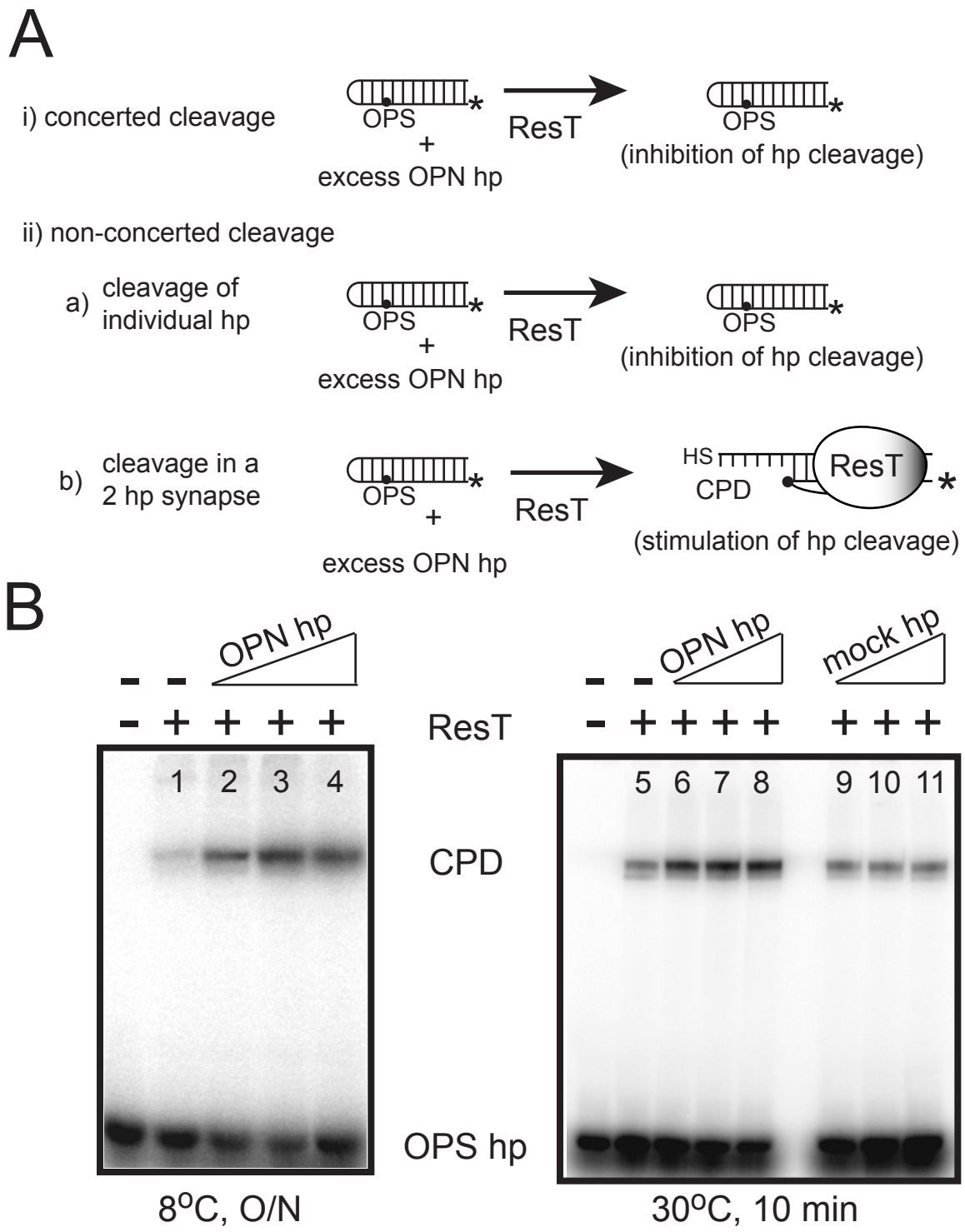

A
i) concerted cleavage
OPS
+
excess OPN hp
ResT
OPS
(inhibition of hp cleavage)
ii) non-concerted cleavage
a) cleavage of individual hp
OPS
+
excess OPN hp
ResT
OPS
(inhibition of hp cleavage)
b) cleavage in a 2 hp synapse
OPS
+
excess OPN hp
ResT
HS
CPD
ResT
(stimulation of hp cleavage)
B
OPN hp
- - - + + + +
1 2 3 4
8°C, O/N
ResT
CPD
OPS hp
OPN hp
mock hp
- - - + + + + + + +
5 6 7 8 9 10 11
30°C, 10 min

Figure 4

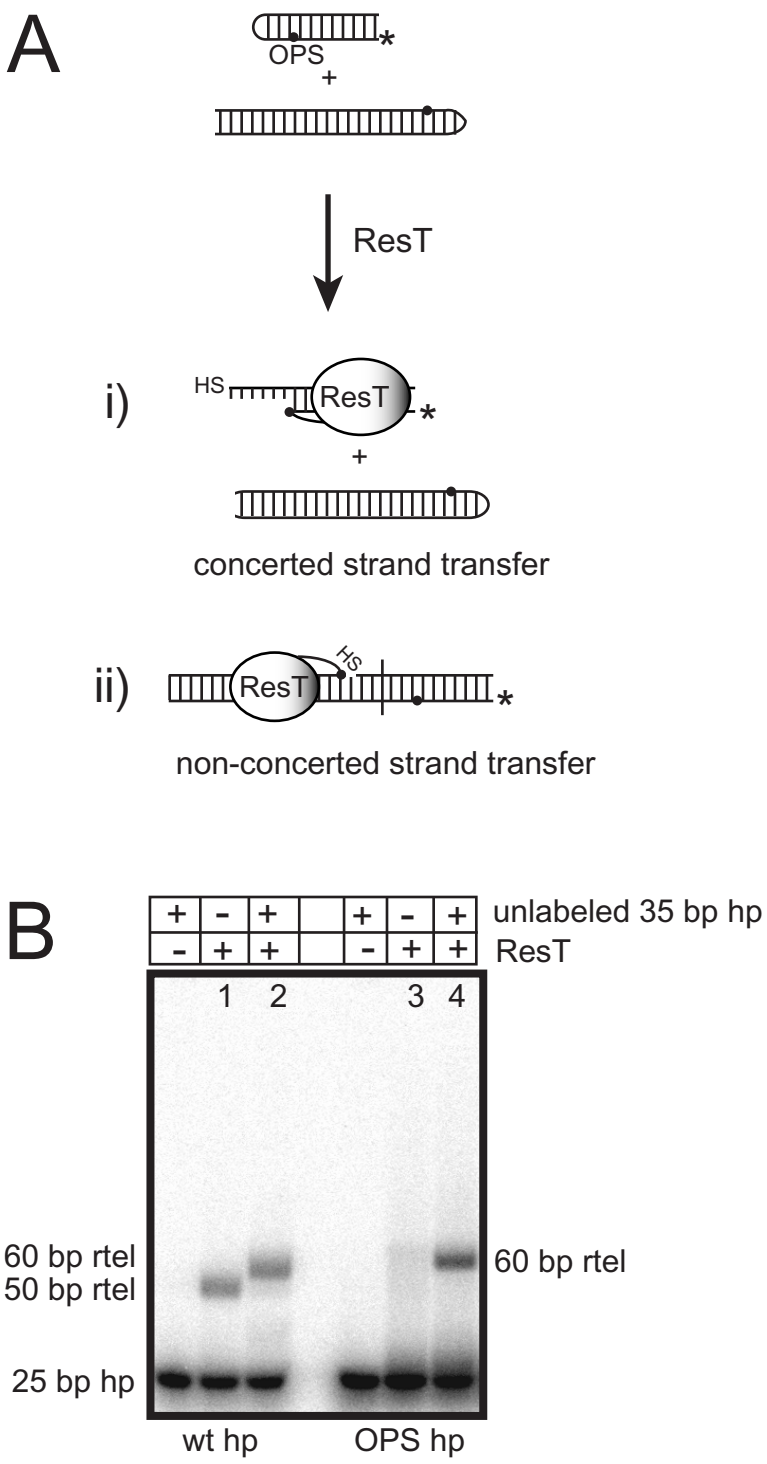

A
OPS
*
+
ResT
i)
HS
ResT
*
+
concerted strand transfer
ii)
ResT
HS
*
non-concerted strand transfer
B
+ - + + - +
- + + - + +
unlabeled 35 bp hp
ResT
1 2 3 4
60 bp rtel
50 bp rtel
25 bp hp
60 bp rtel
wt hp
OPS hp

Figure 5

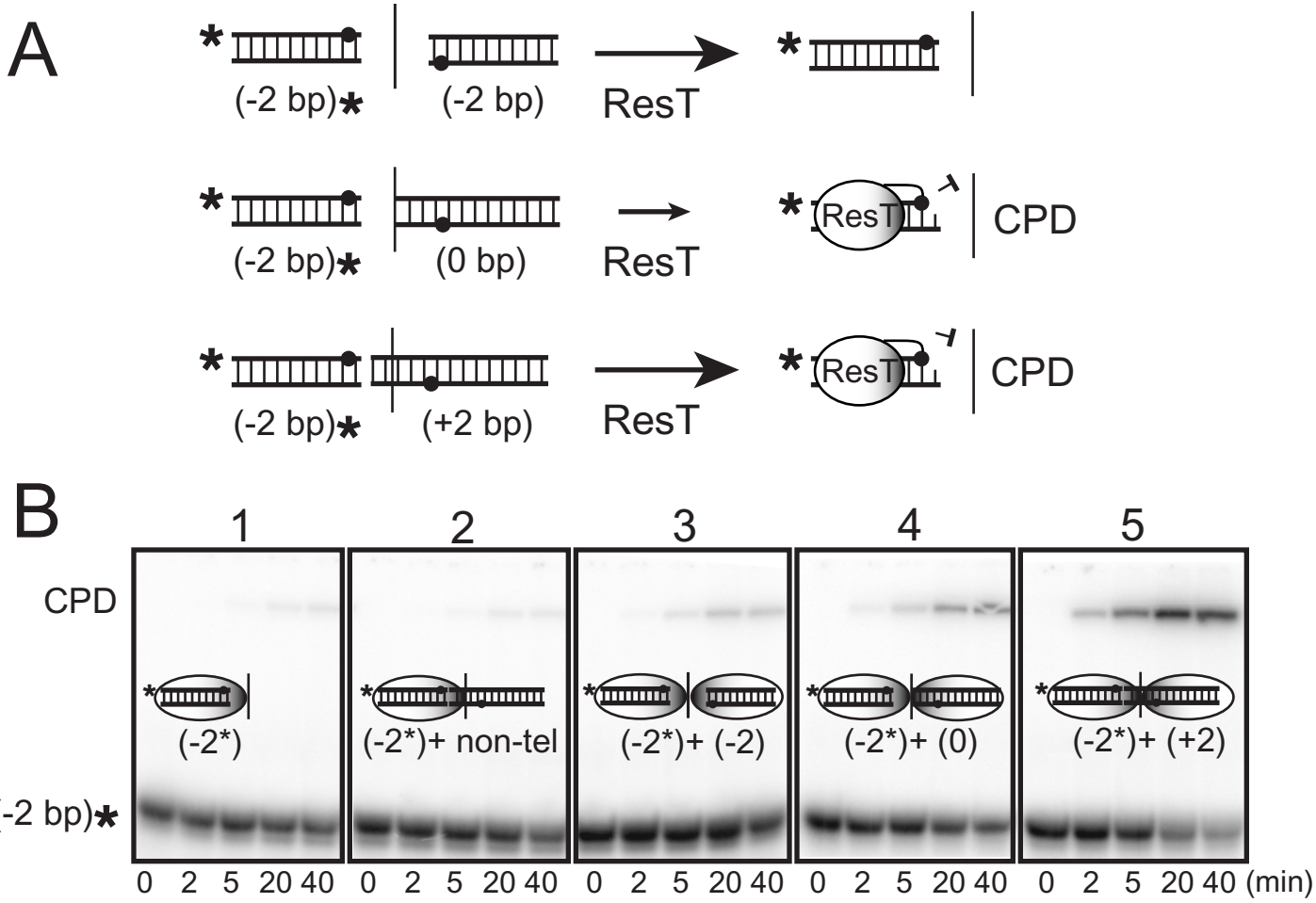

A
(-2 bp)*
(-2 bp)
ResT
(0 bp)
(+2 bp)
CPD
B
1
2
3
4
5
CPD
(-2 bp)*
(-2*)
(-2*)+ non-tel
(-2*)+ (-2)
(-2*)+ (0)
(-2*)+ (+2)
0 2 5 20 40 (min)
C
% cleavage
time (min)
0
10
20
30
40
50
60
5
4
3
1,2

Figure 6