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Tn10 Transposition via a DNA Hairpin Intermediate

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Summary

We present evidence that excision of the nonreplicative transposon Tn10 involves three distinct chemical steps, first-strand nicking, hairpin formation, and hairpin resolution. This three-step mechanism makes it possible for a single protein-active site to cleave two DNA strands of opposite polarity, as appears to be the case in this reaction. We infer the existence of alternating bifunctionality within the active site with suitable modulation of substrate components between steps. DNA double-strand breaks are also made by a "hairpin mechanism" in V(D)J recombination, possibly reflecting the same basic constraints faced in the Tn10 system. Similarities in the basic chemical steps in Tn10 transposition and V(D)J recombination suggest that the V(D)J mechanism may have evolved from a bacterial transposition system.

Introduction

DNA transposition is a form of genetic recombination in which discrete genetic elements, transposons, move from site to site within and between genomes. Transposons are universal components of essentially all organisms and comprise a major source of genetic instability and variation. In bacteria, at least, transposition is an important component in the horizontal transfer of advantageous genes (e.g., those encoding antibiotic resistance). Transposition also plays an integral role in the lytic and lysogenic life cycles of certain bacteriophages (e.g., Mu); similarly, in eukaryotes, retroviral integration is a specialized form of DNA transposition (Berg and Howe, 1989).

All transposition reactions analyzed thus far appear to utilize the same basic chemical strategy for joining transposon ends to target DNA: 3′OH termini are created by hydrolytic nicking and then engage in direct nucleophilic attack upon the two strands of the target DNA in a symmetrical pair of transesterification reactions (Figure 1A) (Mizuuchi, 1997). Transposons fall into two major classes, however, with respect to events at the ends on the two, "nontransferred," strands. In mode 1, the nontransferred strands are also cleaved prior to strand transfer, with the resultant excision of the double-stranded transposon segment from the donor DNA; transposition thus necessarily results in simple insertion of the excised segment at a new site (Figure 1A). This mode is utilized by a number of bacterial transposons, including Tn10, P elements in *Drosophila*, Tc-1 in nematode, and several plant transposons, including Tam1/Tam3. In mode 2, strand transfer occurs without cleavage of the nontransferred strands. In this case, processing of the branched strand transfer intermediate by host enzymes can give either of two outcomes: replicative cointegrate formation, as during lytic growth of bacteriophage Mu and transposition of the bacterial elements Tn1/Tn3, or simple insertion, as during retroviral integration and lysogenic insertion of Mu into its host genome (Berg and Howe, 1989; Saedler and Gierl, 1996).

The experiments reported here address the mechanism of nontransferred strand cleavage during Tn10 transposition. Previous work has shown that excision of this element occurs by successive cleavage of the transferred and nontransferred strands, in obligatory succession, at each end (Bolland and Kleckner, 1995). The resulting excised transposon fragment (ETF) has 3′OH and 5′PO4 termini (Benjamin and Kleckner, 1989). This product could in principle be generated by hydrolytic nicking of both strands at each end (e.g., in a process analogous to type II restriction enzyme cleavage carried out by a pair of symmetrically disposed transposase monomers at each end). Recent findings have suggested, however, that all of the chemical events of Tn10 transposition might be carried out by two transposase monomers, essentially one per end, via a single active site (Bolland and Kleckner, 1996; Kennedy and Haniford, 1996). To accommodate these and other results, it was proposed that excision might occur by a three-step mechanism involving the generation of a hairpin intermediate by transesterification (Figure 1C). First, hydrolytic cleavage of the transferred strand at the terminal nucleotide would generate a 3′OH group. Second, this 3′OH would attack the nontransferred strand, joining the terminal 3′OH to the scissile phosphate to form a hairpin at the transposon end and releasing the flanking donor DNA as a double-stranded end. Third, the hairpin would be resolved by hydrolytic attack at the terminal phosphodiester linkage to regenerate the 3′OH residue at nucleotide 1 of the transferred strand. These steps would be followed by strand transfer. Thus, overall, transposition would involve four reactions in which hydrolysis and transesterification occur in alternating succession, all involving the same critical terminal residues of the transferred strand and thus relatively easily accommodated by a single active site (Bolland and Kleckner, 1996; see Discussion).

The experiments presented below provide several lines of evidence for a DNA hairpin as an intermediate in Tn10 transposition, thus supporting the above model. We discuss the possibility that many transposons that undergo clean excision of the element from the donor site (mode 1 above) might utilize this type of pathway for transposon excision. In addition, since a hairpin intermediate is also formed during V(D)J recombination (McBlane et al., 1995), which mediates rearrangement

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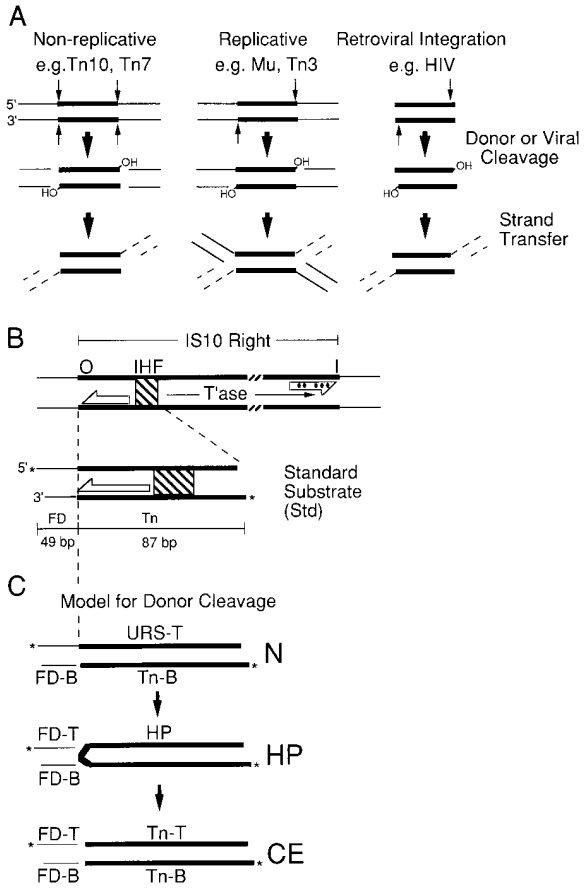

Figure 1. Chemical Steps in DNA Transposition Reactions and Structure of the Standard DNA Substrate Used for Kinetic Analysis of Tn10 Donor Cleavage

(A) The chemical steps in three different modes of DNA transposition are shown. Small vertical arrows indicate positions of DNA strand cleavage. The DNA strands of the transposon that are joined to the target DNA are referred to as the "transferred strands"; the other transposon strands are the "nontransferred strands." Bold, thin, and dashed lines represent transposon (Tn), flanking donor (FD), and target (T) DNA, respectively.

(B) Illustration of IS10 Right, which contains an outside (O) and inside (I) end, and the "standard" DNA substrate (Std) used in this work. The open arrow and striped box represent Tn10/IS10 transposase and integration host factor (IHF) binding sites, respectively. Each strand of the Std substrate is 132 nt in length, and there is a 4 nt 5′ overhang at each terminus. The asterisk (*) indicates the presence of a ^{32}P label.

(C) Model for donor cleavage (see details in text). N, HP, and CE are nicked, hairpin, and cleaved end DNA species, respectively. URS is unreacted substrate. T and B refer to top and bottom strands, respectively.

of antibody and T cell receptor coding segments, these findings suggest that the chemistry of phosphoryl transfer reactions provides a unity that underlies the apparent diversity of many biologically important DNA breakage and joining reactions.

Results

Detection and Kinetic Competence of the Hairpin Intermediate

A kinetic analysis of the Tn10 donor cleavage reaction in vitro has been performed using an assay that utilizes a short linear DNA fragment carrying a Tn10 end as substrate (Sakai et al., 1995). The substrate DNA is terminally labeled at the 5′ ends with ^{32}P. As described in previous studies, assembly and progression of protein/DNA transpososomes can be monitored by nondenaturing gel electrophoresis of unprocessed reaction mixtures; in addition, following DNA extraction, chemical changes in the substrate DNAs can be monitored in native and/or denaturing gels (Bolland and Kleckner, 1995; Sakai et al., 1995; see Experimental Procedures).

When transposase, IHF, and a standard substrate fragment (Std; Figure 1B) are incubated in buffered salt solution lacking divalent metal ion, the components assemble stably into the first protein/DNA complex of the transposition reaction, the "paired ends complex" or "PEc," which contains two intact transposon end fragments. Upon addition of $MgCl_2$, the chemical steps of the donor cleavage reaction begin within these assembled complexes. Kinetic analysis was performed on such a reaction by removing samples at various time points after $MgCl_2$ addition and mixing with phenol to stop the reaction. The DNAs in these samples were purified and then analyzed by denaturing gel electrophoresis with the levels of various observed species quantified by phosphorimaging.

The results of such an analysis are shown in Figure 2. The model in Figure 1C predicts the appearance of three species in addition to unreacted substrate, URS. One species is the transposon bottom-strand product, Tn-B, generated by first-strand nicking; this species is expected to appear early, decrease in level as it is converted to hairpin, and then increase in level due to second-strand nicking via hairpin resolution. The other two species are the hairpin itself, HP, which should appear and disappear as a transient intermediate, and the flanking donor top-strand product (FD-T), which is a stable byproduct of hairpin formation and thus should accumulate over the course of the experiment. Three new species of appropriate molecular weight are observed (Figure 2A) and exhibit appropriate kinetics (Figure 2B). Specifically, Tn-B appears first; HP and FT-D then appear, coordinately; HP then disappears at later time points while FD-T continues to accumulate; and Tn-B also continues to accumulate.

Appropriate analysis of these data permits a quantitative description of the kinetics of the three steps of Tn10 excision: first-strand nicking, hairpin formation, and hairpin resolution (Figure 2C). The nicked form appears first and then disappears; the lifespan of this species is approximately 30 min. The transposon hairpin appears next and disappears; the hairpin lifespan is approximately 15 min. The final product, the cleaved end, appears latest and accumulates continuously with time. Interestingly, under these conditions at least, hairpin formation is substantially slower than hairpin resolution. Since a significant rearrangement of terminal DNA would be required for hairpin formation (Figure 1C), these kinetics might signal the existence of a particularly difficult conformational change in the transpososome.

Further analysis yields cumulative curves that describe, for each step, how many molecules have completed that step, or achieved that stage, as a function of time (Figure 2D). The results show directly that the three inferred stages occur in appropriate succession:

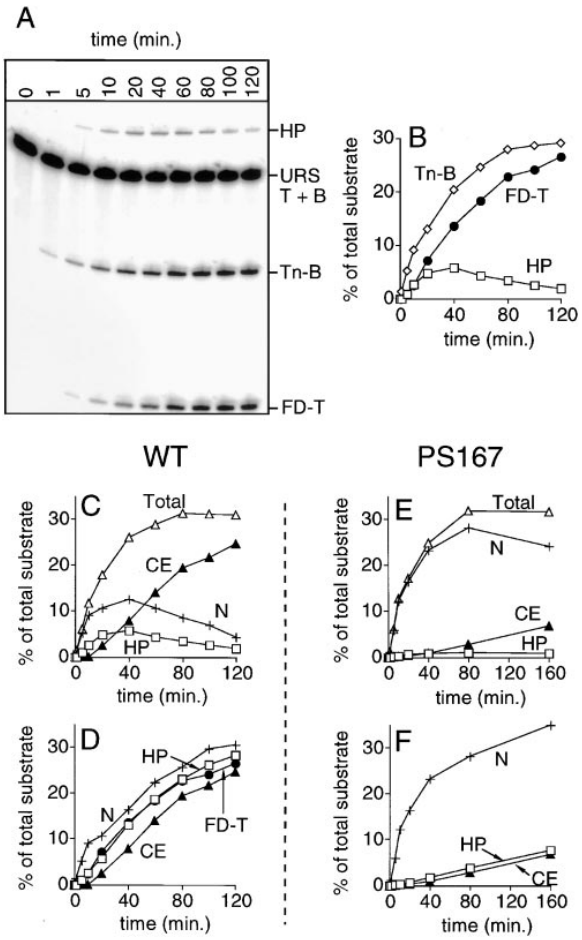

Figure 2. Donor Cleavage Time Course

Paired ends complexes (PEcs) were assembled using either wild-type or PS167 transposase proteins and ^{32}P (5′) end-labeled standard substrate in the absence of a divalent metal ion (see Experimental Procedures). Donor cleavage was initiated by adding $MgCl_2$, and aliquots were removed at the indicated time points and the reactions stopped by phenol extraction. For analysis, purified DNA was applied to a 5% denaturing polyacrylamide gel.

(A) A wild-type time course is shown. HP, Tn-B, and FD-T are detected in addition to URS T+B (described in Figure 1C).

(B) The relative amounts of Tn-B, HP, and FD-T from (A) were quantified by phosphorimaging, expressed as percent of total substrate, and plotted as a function of time.

(C) The levels of nicked (N) and cleaved-end (CE) species were calculated from the data in (B) as described in Experimental Procedures and plotted along with HP and the total amount of reacted substrate (N+HP+CE).

(D) Cumulative curves corresponding to the relative number of molecules that have entered a particular stage have been calculated for N and HP and plotted along with CE and FD-T (see Experimental Procedures).

(E) Levels of HP, N, and CE from the PS167 donor cleavage reaction are shown.

(F) Cumulative curves for N and HP formed in the PS167 reaction have been calculated and plotted along with CE as in (D). The fact that the cumulative curve for N exceeds the total amount of substrate converted (see curve) is likely due to an underestimate of the lifespan of N from (E).

first-strand nicking is followed by hairpin formation and release of flanking donor DNA, which are contemporaneous, followed in turn by formation of the cleaved-end product. Approximately 30% of substrate was converted to PEc in this experiment (data not shown).

None of the three species, including the putative hairpin, is observed in reactions carried out with a mutant transposase, DA97, shown previously by Bolland and Kleckner (1996) to be catalytically inactive for nicking, excision, and strand transfer, indicating that first-strand nicking is required for hairpin formation (data not shown).

The kinetics of Tn10 excision has been investigated previously in similar experiments. These studies revealed the temporal order of transferred and nontransferred strand cleavage (Bolland and Kleckner, 1995).

The New Intermediate Has Molecular Properties Predicted for the Proposed Transposon End Hairpin

The new intermediate revealed by kinetic analysis of Tn10 excision has almost exactly the same mobility as the cleaved-end species on a native gel. A direct comparison between the putative hairpin purified from a denaturing gel and the cleaved-end species purified from a native gel shows that the two species comigrate (Figure 3A).

Furthermore, in a two-dimensional gel (Figure 3B) where DNA species are first separated under nondenaturing conditions and then separated under denaturing conditions, all of the predicted species, including the hairpin, are revealed by appropriate behavior. The cleaved-end species migrates with the appropriate mobility in the first dimension and with a much faster mobility in the second dimension, as expected for a simple duplex molecule that separates into component single strands upon denaturation. Another species migrates at about the same position as the cleaved-end molecule in the first dimension but then migrates with a reduced mobility in the second dimension; such behavior is uniquely diagnostic of a hairpin molecule, as its length will be doubled by denaturation. Finally, species that migrate as unreacted substrate in the first dimension resolve in the second dimension into the corresponding faster-migrating intact single strands (URS T+B) plus a shorter fragment of the length expected for the bottom-strand transposon segment diagnostic of transferred strand nicking (Tn-B); the corresponding bottom-strand flanking donor segment is not detected because it carries no label. Importantly, there is no fragment that migrates with unreacted substrate in the first dimension and with the mobility of flanking DNA (FD-T) after denaturation; instead this species is released only from a form that runs in the first dimension at the position of the duplex flanking donor segment (i.e., from a molecule generated by double-strand cleavage at the end). These findings confirm that top-strand cleavage only occurs after bottom-strand cleavage.

The putative hairpin species also is sensitive to cleavage by single strand–specific nucleases at phosphodiester bonds expected to be in the tip of the hairpin (Figure 3C). The putative hairpin species was purified from a denaturing gel and then treated with different amounts of either mung bean nuclease (lanes 1–3), P1 nuclease (lanes 6–8), or left untreated (lane 4). Reactions were analyzed on a denaturing gel alongside purified Tn-B (lane 5) and an appropriate DNA sequence ladder

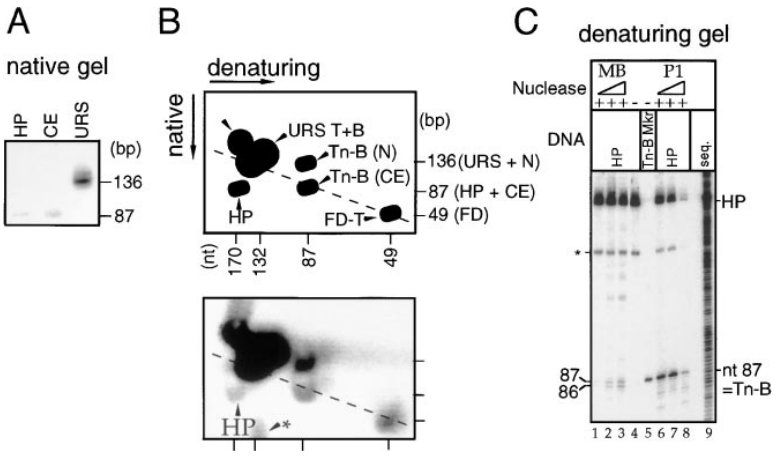

Figure 3. Additional Evidence for Hairpin Formation

(A) DNA hairpin, generated as in Figure 2A, was isolated from a 5% denaturing polyacrylamide gel and applied to a native 5% polyacrylamide gel alongside purified CE and URS.

(B) A reaction equivalent to the 40 min time point in Figure 2A was subjected to two-dimensional electrophoresis, with electrophoresis in the first dimension being through a native 5% polyacrylamide gel and through an 8% denaturing gel in the second dimension. The cartoon shows that 136 (URS and N), 87 (HP and CE), and 49 (FD) bp species are separated in the first dimension, and 170 (HP), 132 (URS T+B), 87 (Tn-B [CE] and Tn-B [N]), and 49 (FD-T) nt species are separated in the second dimension. Only the positions of hairpin and unreacted substrate (indicated by *), which come from loading of the above reaction, and pure standard substrate in separate lanes of the first dimension gel are indicated in the autoradiogram. The species indicated by the unlabeled arrow in the upper panel may be URS molecules that are not fully denatured.

(C) Purified DNA hairpin was treated with 1, 5, and 10 U of mung bean nuclease (MB), lanes 1–3 respectively, or 0.009, 0.018, and 0.072 U of P1 nuclease, lanes 6–8, respectively, or left untreated, lane 4. Reactions were terminated by phenol extraction and analyzed on a 5% denaturing polyacrylamide gel alongside purified Tn-B (lane 5) and a dideoxy-sequencing ladder (mixture of G, A, T, and C reactions) made using the standard substrate as template and an appropriate primer. The appearance of URS T+B (indicated by *) in reactions with purified hairpin is due to contamination of the hairpin preparation with this species.

(lane 9). P1 digestion yields a single major cleavage product that exactly comigrates with Tn-B. Mung bean digestion is less efficient and less specific; however, the two primary cleavage products comigrate with Tn-B and at a position one nucleotide shorter than Tn-B, respectively. These cleavage patterns correspond qualitatively to what might be expected a priori, and they also match exactly the patterns predicted for a self-complementary oligonucleotide containing the sequence 5′ CAGCTG 3′ at a hairpin tip (Kabotyanski et al., 1995).

Finally, DNA sequence analysis of the putative transposon-end hairpin species confirms its identity and also demonstrates that for the majority of hairpin molecules no nucleotides have been either lost or added during their formation (Figure 4). The putative hairpin species was purified from a denaturing gel and then subjected to treatment with DMS plus piperidine (i.e., by the Maxam and Gilbert DNA sequencing cleavage reaction specific to G residues). The observed G cleavage pattern matches very well with the pattern expected from the predicted sequence of the hairpin. In addition, a moderate degree of cleavage occurs at the predicted C position in the tip of the hairpin (Figure 4 and data not shown). This is not unexpected because unpaired C residues are sensitive to modification at the N3 position by DMS and this leads to cleavage by piperidine (Nadel et al., 1995).

Preformed Hairpins Are Cleaved by Transposase

Hairpin DNA molecules having the same structure as those arising during the transposition reaction were prepared synthetically (Figure 5A; see Experimental Procedures). These artificially prepared molecules assembled into the PEcs in the absence of divalent metal ion with an efficiency comparable to that of the normal linear DNA substrate (Figure 5B, compare lanes 1, 3, and 5). Furthermore, when $MgCl_2$ is added to reactions containing assembled PEcs, the hairpin undergoes nicking at precisely the nucleotide position of nontransferred strand cleavage during the standard transposition reaction. For the preformed hairpin substrate such a nick should yield a 65 nt Tn-B species plus an unlabeled complementary strand, Tn-T, that is two nucleotides shorter. Prior to $MgCl_2$ addition, a small amount of both Tn-B and labeled Tn-T are present as a consequence of the way the hairpin was made (Figure 5C; Experimental Procedures). As the reaction proceeds, the level of Tn-B increases over time, while the level of the Tn-T species remains unchanged (Figure 5C, lanes 5–10). Appearance of the nicked species is absolutely dependent upon catalytically active transposase (Figure 5C, lanes 3–4).

Nicking of the preformed hairpin occurs with kinetics and efficiency very similar to those of the "native" hairpin (i.e., the hairpin formed in the standard reaction). Roughly 80% of preformed hairpins are nicked in 20 min or less (Figure 5C) as compared to >90% for the native hairpin (this latter value is based on the calculated average lifespan of 15 min for the native hairpin [Figure 2C]). Moreover, the final efficiency of hairpin nicking, per PEc present prior to $MgCl_2$ addition, is very similar in the two cases, ~75% with the native hairpin (level of CE at t = 120 min, Figure 2C) and ~65% with the preformed hairpin substrate (calculated from the amount of substrate converted to PEc, ~5%, and the amount of substrate converted to Tn-B, ~3%, in Figures 5B and 5C, respectively).

These results suggest that the preformed hairpin is accepted by transposase protein as an essentially normal molecule both for assembly and for cleavage, which exhibits all of the same features and dependencies as second-strand cleavage in the standard reaction. The normal efficiency and kinetics of this process contrast strikingly with the aberrant kinetics of second-strand nicking observed in a situation where hairpin formation should be blocked and cleavage likely occurs entirely by hydrolysis (below). The ease with which a hairpin

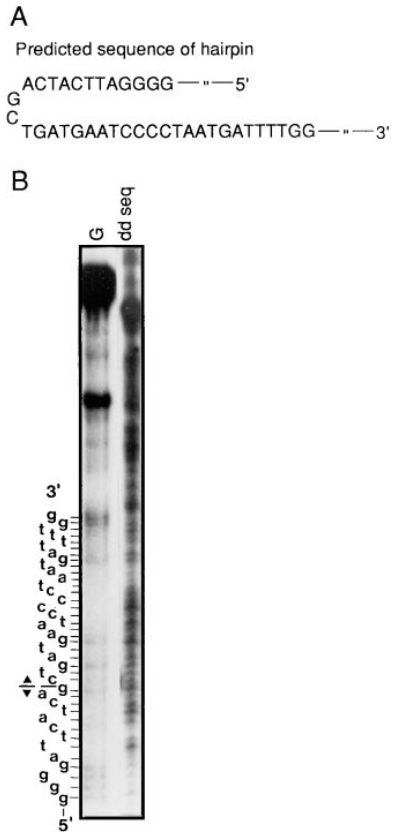

Figure 4. DNA Sequence Analysis of the Hairpin

(A) The predicted sequence of a portion of the DNA hairpin formed in reactions with the standard substrate is shown.

(B) Purified DNA hairpin was subjected to the Maxam and Gilbert G-specific cleavage reaction and analyzed on an 8% denaturing gel. The sequence shown alongside the gel was inferred from the position of G-specific cleavages and the spacings between G cleavage products, which were determined from the dideoxy sequencing ladder. The ladder was made as in Figure 3C. The dashed horizontal line and oppositely oriented arrows indicate the "turn-around-point" of the DNA hairpin.

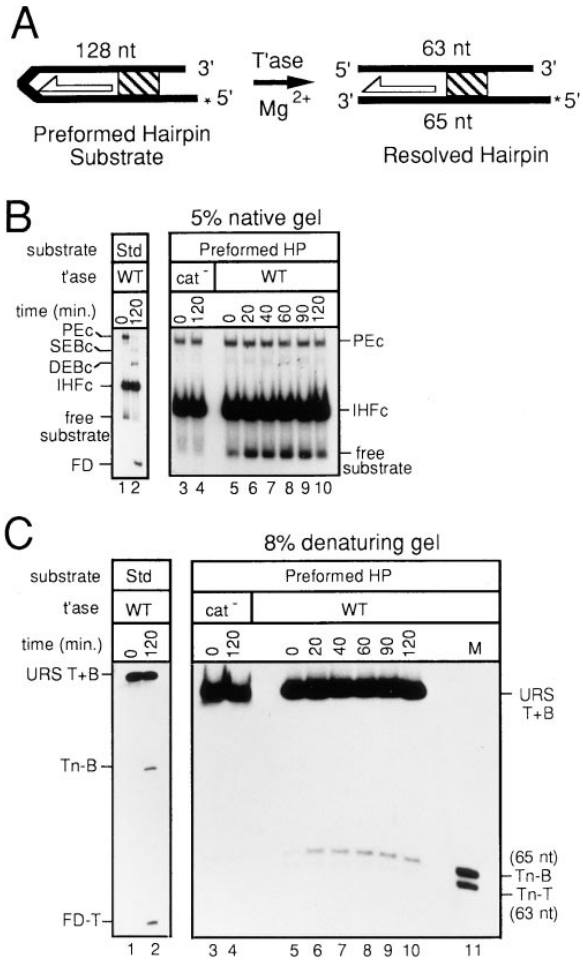

Figure 5. WT Transposase Converts a Preformed Hairpin to a Cleaved-End Form

A preformed hairpin (shown in [A]) was assembled into PEc with either wild-type transposase or DA97 (catalytic$^-$) transposase in the absence of a divalent metal ion. Reactions were divided into aliquots, and $MgCl_2$ was added to each (except for the "0" time point) for the indicated amounts of time. Each aliquot was then further divided into two equal portions for analysis on either a native gel (B) or a denaturing gel (C). For the former, samples were loaded without prior treatment with a denaturant, and for the latter samples were phenol extracted before loading. For comparison purposes, the equivalent experiment was also performed with wild-type transposase and the standard (Std) substrate. The markers (M) in lane 11 of (C) are 65 nt and 63 nt fragments, corresponding to Tn-B and Tn-T, respectively (see diagram in [A]). During the preparation of the preformed hairpin, low levels of Tn-B and Tn-T were generated (see Experimental Procedures), explaining the presence of these species in all of the relevant lanes in (C). In (B), the level of PEc decreases only in the reaction with the Std substrate. This is because removal of flanking DNA from one or both ends generates single end break complex (SEBc) and double end break complex (DEBc), respectively, which have significantly different mobilities as compared to the PEc. In contrast, cleavage of the preformed hairpin PEc results only in the opening of the hairpin and is therefore not expected to cause a significant change in mobility.

substrate can be utilized at all relevant steps of the process provides strong support for a hairpin as a true intermediate in the normal transposition reaction.

Previous work has shown that DA97 transposase is defective in each observable step of the reaction as assayed individually (i.e., in first-strand nicking of duplex substrates, in second-strand nicking of substrates already carrying a nick on the transferred strand, and in strand transfer of substrates precleaved at the transposon termini). These findings implied that all of the chemical steps of transposition utilize at least some common catalytic active-site residues (Bolland and Kleckner, 1996). The finding that DA97 transposase fails to promote nicking of a hairpin substrate provides the final link in this chain of evidence.

Substitution of a 3′H for a 3′OH at the Nicked Transposon Termini Eliminates Both Detectable Hairpin Formation and Timely and Efficient Second-Strand Cleavage

According to the proposed model, formation of the hairpin intermediate and thus, indirectly, second-strand nicking, are dependent upon nucleophilic attack by the 3′OH group exposed by the initial transferred-strand nick. This dependence has been examined by comparing the behavior of two substrates, one of which contains

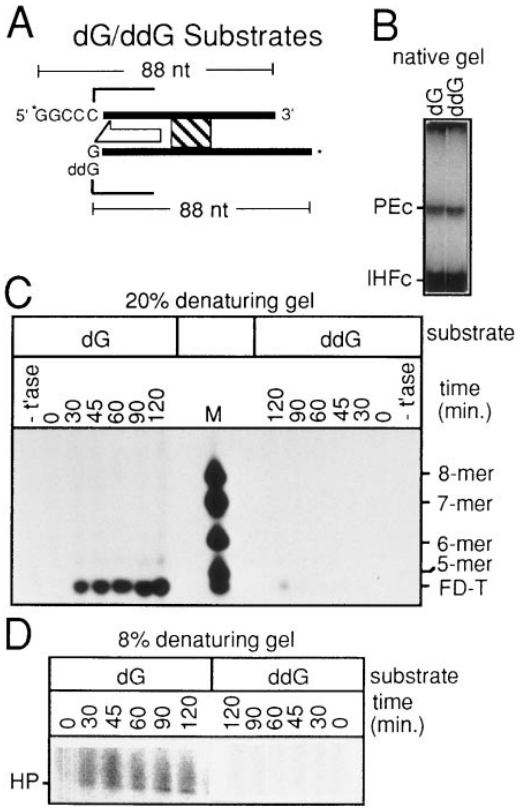

Figure 6. A ddG at the Terminal Nucleotide of the Transferred Strand Inhibits Hairpin Formation and Second-Strand Nicking

dG and ddG substrates, labeled at their 5′ termini with ^{32}P (A), were assembled into PEcs in the absence of a divalent metal ion. A portion of each reaction was analyzed on a 5% native gel (B) to investigate the levels of PEc formed. $MgCl_2$ was added to the remainder of each reaction, and reactions were allowed to proceed for the indicated times whereupon they were terminated by the addition of EDTA to 10 mM and divided for analysis on 20% (C) and 8% (D) denaturing gels. The former was used to investigate second-strand nicking, which should result in the release of the ^{32}P labeled, 4 nt donor fragment (5′ GGCC 3′). The latter was used to investigate hairpin formation. Markers (M) in (C) include the 4-mer sequence, 5′GGCC. In (C) and (D), only the portions of the gels containing the relevant species are shown.

a deoxyG at nt 1 of the transferred strand and the other contains a dideoxyG at this position (Figure 6A). The dG substrate should undergo hairpin formation and second-strand nicking; the ddG substrate, in contrast, should be defective in hairpin formation and should not undergo any nicking on the nontransferred strand.

These predictions are fulfilled. The dG and ddG substrates both form transpososomes efficiently (Figure 6B). Then, upon addition of $MgCl_2$, the dG substrate undergoes hairpin formation and relatively rapid and efficient nicking of the top strand (Figures 6C and 6D). The ddG substrate, in contrast, exhibits no detectable hairpin formation and only a very low level of nicking, with less than 5% the level observed for the dG substrate even by the latest time point, 120 min. In the context of the proposed model, this low level of nicking can be explained by saying that the normal reaction pathway is blocked and that, very slowly and/or inefficiently, second-strand nicking occurs by an aberrant process.

PS167 Mutant Transposase Is Specifically Defective in the Hairpin Formation Step of Ends Cleavage

If a hairpin occurs as a normal intermediate in Tn10/IS10 transposition, particular mutant transposases should be specifically defective in hairpin formation and/or resolution. A previously identified mutant, PS167, appears to be one such mutant (Haniford et al., 1989). In reactions carried out with PS167 transposase, the PEc forms with normal efficiency (data not shown). Once $MgCl_2$ is added to these assembled PEcs, first-strand nicking occurs efficiently. Instead of turning over, however, the nicked product accumulates to a high level, with a corresponding deficit of both hairpin and cleaved-end forms. The total fraction of PEcs that undergo at least the first chemical step is the same as in the wild-type reaction, however, indicating inhibition after first-strand nicking (Figure 2E). Cumulative curves confirm these conclusions: PEcs enter the nicked stage with normal kinetics, while entry into hairpin and cleaved-end stages is severely delayed and inefficient (Figure 2F).

Interestingly, however, hairpin resolution occurs normally in this mutant: the lifespan of the hairpin intermediate in the mutant (calculated from Figure 2E) and wild-type reactions are ~17 and ~15 min, respectively. Thus, PS167 transposase is specifically defective in the hairpin formation step of excision.

Previous work has shown that PS167 transposase is also defective in strand transfer (Haniford et al., 1989). Thus, this mutation specifically affects the two transesterification steps of the transposition reaction, while having no discernible effect on the two hydrolytic steps. Since the strand transfer defect is attributable at least in part to a defect in stable target capture (Junop and Haniford, 1997), the PS167 mutation may be defective in a conformational change following hairpin resolution. The same effect could explain defective hairpin formation, which also seems to involve an important conformational change (above). Interestingly, too, the PS167 defect in transposon ends cleavage is not observed on supercoiled DNA (Haniford et al., 1989), which implies a postsynaptic role for supercoiling in driving this chemical step. This finding fits nicely with a recently proposed model for geometric, torsional, and topological changes in the transpososome/DNA complex during the early steps of Tn10 transposition (Chalmers et al., 1998).

Discussion

Tn10 Excision Occurs via a Hairpin Intermediate

In this work we show that a DNA hairpin is formed at Tn10 ends during the transposon excision reaction. Several lines of evidence argue that this hairpin species is an obligatory intermediate in the donor cleavage process, thus supporting the three-step model for donor cleavage presented in Figure 1C. We have also identified a transposase mutant that is specifically defective in formation of the hairpin intermediate.

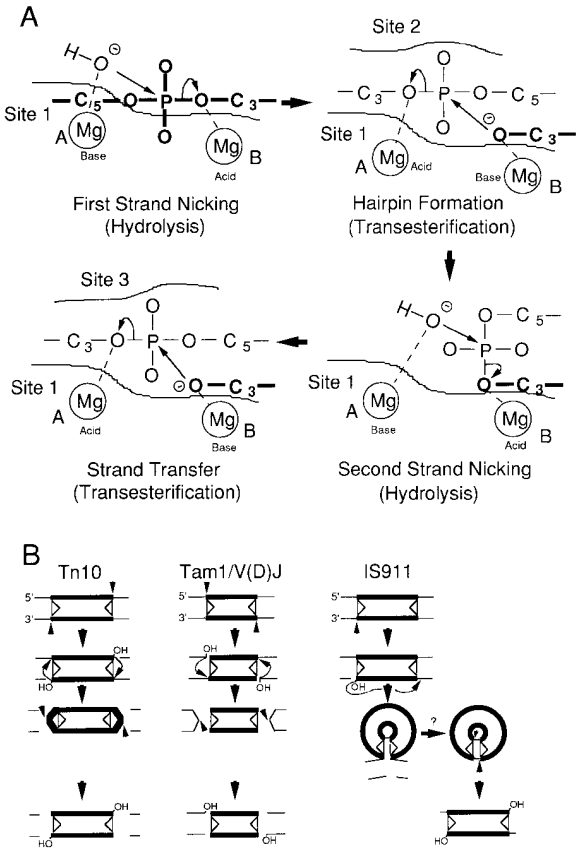

Figure 7. Models for Double-Strand Break Formation

(A) A two–metal ion mechanism for catalysis of the four chemical steps in Tn10 transposition. DNA strands are represented by a straight line, and only the scissile phosphate is shown in detail; the transferred strand is shown in bold. The two Mg^{2+} binding sites are designated A and B, and dotted lines indicate Mg^{2+} interactions with ligands; only a subset of possible interactions is shown. Site 1 is the catalytic core of transposase that includes the "DDE motif." Sites 2 and 3 are segments of transposase that are responsible for bringing the nontransferred strand and target DNA, respectively, into the active site. The critical aspects of this model are as follows: (1) The catalytic core domain stays in contact with the transferred strand throughout all of the chemical steps; (2) the Mg^{2+} in site A and B alternate between roles as general acid and general base catalysts at each step in the reaction; (3) different segments of transposase guide different substrates into the active site in a precise order that is presumably governed by conformational changes in transposase at each transition point.

(B) A comparison of the chemical steps required to generate double-strand breaks in four different systems. Thick and thin lines represent transposon and flanking donor DNA sequences, respectively, and triangles represent inverted repeat sequences. For the V(D)J reaction, thick and thin lines represent signal and coding sequences, respectively. The question mark in the IS911 panel indicates that it has not yet been established how the indicated step occurs.

The Hairpin Mechanism Permits a Single Protein Monomer with a Single Active Site to Make a Double-Strand Break in DNA

Advantages of the Four-Step Mechanism

Tn10 transposase carries out four distinct chemical reactions in Tn10 transposition: first-strand nicking, hairpin formation, hairpin resolution (or second-strand nicking), and strand transfer. The available evidence argues that a single active site contributed from a single transposase monomer (per transposon end) is used repeatedly to catalyze all four steps (Bolland and Kleckner, 1996, and above). This is spatially sensible for two reasons. First, all four reactions directly involve the residue at the 3′ termini of the transposon segment. Second, while the two hydrolysis reactions have the same overall effect of cleaving single strands of opposite polarity, formation of the hairpin can bring the second (top) strand down into a junction with the bottom strand such that the bond to be cleaved lies within a strand region having the same local polarity as during the first-strand nicking.

Alternating Bifunctionality within the Catalytic Active Site

Another striking feature of the Tn10 transposition reaction is that the four steps involve the occurrence of alternating hydrolysis and transesterification reactions, two of each. This feature implies that the single active site has the unique property of being able to exist alternatively in either of two configurations. One configuration promotes strand cleavage: a water molecule attacks the phosphodiester bond adjacent to the 3′ terminal residue. This bond is located within the intact transposon for first-strand nicking and within the hairpin for second-strand nicking. The second configuration promotes strand joining: the 3′ terminal hydroxyl generated in the prior hydrolysis step attacks the phosphodiester bond of another DNA strand. During hairpin formation this other strand is the complementary strand at the transposon end; during strand transfer the other strand is a component of the target DNA duplex. One way of achieving alternating hydrolysis and transesterification reactions within a common active site has been suggested by studies on alkaline phosphatase and group 1 and 2 self-splicing introns, the "two metal ion hypothesis" of Steitz and Steitz (1993). The complex alternating bifunctionality required for Tn10 transposition can be explained by combining this type of mechanism with appropriate changes in the nature and configuration of the DNA/protein components at various stages, as illustrated in Figure 7A.

An interesting variation on the Tn10 theme is provided by bacteriophage Mu, where transposon end nicking and ensuing strand transfer are carried out by the same active site (Baker and Luo, 1994). It has been shown that the Mu transpososome contains four MuA monomers and thus the potential for four active sites per transpososome (Lavoie et al., 1991). Recent evidence suggests, however, that two of these monomers do not donate DDE motif residues for the catalytic steps as shown for Tn10, and thus the same active site is utilized repeatedly for both nicking and strand transfer reactions (Namgoong and Harshey, 1998; T. Baker, personal communication). Interestingly, the Mu active site appears to be composed of domains from different MuA monomers (Yang et al., 1995). It will be interesting to determine whether this same feature applies to Tn10.

How Other Transposons Carry out Double-Strand Excision

Tn5 and Tn7 are other examples of bacterial transposons that transpose by a nonreplicative mechanism. Tn5

is a close relative to Tn10 and behaves similarly in many respects examined thus far (Goryshin and Reznikoff, 1998 and references therein); it seems likely that the correspondence will extend to the mechanism of excision as well. Tn7 differs from Tn10 and Tn5, however, in that it encodes multiple proteins that are required for its transposition (Bainton et al., 1993). Mutational studies have shown that the Tn7 transposase exists as a heteromeric complex of an as yet undefined number of TnsA and TnsB molecules; mutations in TnsA can block nontransferred strand cleavage while mutations in TnsB can block both transferred strand cleavage and strand transfer, and both TnsA and TnsB proteins must be present to see any of these activities (May and Craig, 1996; Sarnovsky et al., 1996). These findings have led to the suggestion that TnsA and TnsB each contain a single active site and that, during Tn7 excision, each protein is responsible for cleaving a separate DNA strand at a transposon–donor junction. In light of the current data presented in this work, an alternative possibility presents itself: double-strand break in Tn7 excision could involve a hairpin if the organization of the heteromeric transposase is such that TnsA and TnsB cooperate to form a single composite active site or if TnsA is strictly an allosteric modulator of TnsB. A hairpin intermediate has not been found in this system, however (N. Craig, personal communication).

IS911 is another transposable element that forms simple inserts via a single transposase protein, OrfAB (Polard et al., 1992). Excision of this element seems superficially to be rather unique because it involves a circular intermediate (Ton-Hoang et al., 1997). But further inspection suggests that the mechanism is simply a variant of the hairpin mechanism with, once again, hydrolytic and transesterification steps occurring in alternation (Figure 7B). In the first step, IS911 undergoes the usual initial first-strand nicking but at only one end of the element. The single 3′ hydroxyl terminus then attacks the other end of the element, in an intrastrand transesterification reaction. The resulting intermediate is then converted to a covalently closed transposon circle by some as yet undefined pathway. In this transposon circle, the new junction sequence consists of opposing IS911 termini separated by 3 bp. The IS911 transposase binds to and nicks both transposon ends leaving 3′OH transposon termini, presumably using reaction chemistry identical to that used in the first step. Because the nicks are close together and on opposite strands, the circle is linearized and then the free 3′OH transposon termini are available for attack of a target site. The remaining flanking DNA would be removed by host enzymes to complete the process. IS2 also utilizes this same strategy (Lewis and Grindley, 1997).

While Tn10 is the only bacterial transposon that has been shown to solve the double-strand excision problem by utilizing a hairpin mechanism, there is evidence that some eukaryotic transposons use a similar strategy. For example, excision of the Tam1 and Tam3 transposons of *Antirrhinum majus* leaves donor DNA footprints, which can be explained by a hairpin mechanism, as proposed many years ago by Coen et al. (1989). As shown in Figure 7B, the main difference in this mechanism compared to Tn10 would be that the initial nicks are made at the junctions between the nontransferred strands and flanking DNA, and as a result the hairpins would be formed in the donor DNA. In this case the transposase would not have to resolve the hairpin for the transposition reaction to proceed to the next step.

Complex Double-Strand Cleavage Reactions in Other Systems

V(D)J Hairpins

V(D)J recombination provides another example of a system that uses a hairpin mechanism to make a double-strand break in DNA (see Figure 7B). Both Rag1 and Rag2 proteins are necessary for nicking and hairpin formation, and the Rag1/Rag2 recombinase can catalyze a strand transfer reaction in which a signal strand is joined to a coding strand (McBlane et al., 1995; Melek et al., 1998). While this reaction does open up the coding end hairpin, it does not give rise to the appropriate end product that is the joining of two coding sequences. It is not yet known what protein is responsible for opening up the coding end hairpins in the pathway that leads to the formation of correct end product. Interestingly, exposure of the 3′OH termini in the signal sequence by the hairpin mechanism and subsequent joining via transesterification to another DNA strand are equivalent to the steps in the Tn10 transposition reaction. In fact, recent studies have demonstrated that both 3′OH termini of an excised signal end fragment can be joined to a single short target sequence in what is effectively a transposition reaction (Agrawal et al., 1998; Hiom et al., 1998). Perhaps the V(D)J system evolved from a transposon, or vice versa.

Restriction Enzymes with Asymmetric Binding Sites

Another unusual type of double-strand cleavage is represented by FokI endonuclease, where a monomer of the enzyme binds an asymmetric DNA site but then promotes staggered cleavages of the two strands at a distance from the recognition site. FokI is comprised of a DNA-binding domain that engages the recognition site and a catalytic domain that is tethered to the DNA-binding domain via a flexible linker domain. It has been suggested that once a FokI monomer binds its recognition site and nicks one strand of the cleavage site, a conformational change takes place in the linker region, the net result of which is to reposition the catalytic domain so that it can cleave the scissile phosphate of the opposite strand (Wah et al., 1997). It is tempting to wonder whether a hairpin mechanism might not also be utilized in this case.

Concluding Remarks

A variety of proteins have now been identified that make double-strand breaks by mechanisms more complex than the symmetrical nicking reactions promoted by type II restriction endonucleases. One important mechanism involves the formation of a DNA hairpin intermediate. This strategy is employed in prokaryotes in Tn10 transposition, in eukaryotes in V(D)J recombination and evidently in Tam1 and Tam3 transposition, and likely in quite a number of other cases. In the Tn10 case, at least, the entire process appears to be carried out by a single

active site. It will be interesting to see what other systems employ the hairpin mechanism to deal with the double-strand break problem and whether there are alternative mechanisms for solving this problem that differ from those already described here.

Experimental Procedures

In Vitro Transposition Reaction

DNA Substrates

The standard transposon end substrate fragment (Figure 1B, Std) is described in Sakai et al. (1995). All substrates used in this work were 5′ end labeled using polynucleotide kinase (NEBL) and [γ-^{32}P]ATP (6000 Ci/mmol [ICN]).

The preformed hairpin substrate was prepared by digestion of the miniTn10 plasmid, pNK3287, with PvuII, which gives rise to a 2140 bp mini-Tn10 fragment and a larger donor backbone fragment. The digest was treated with T4 DNA ligase to create mini-Tn10 circles with self-ligated termini (among other products), and the resultant mixture was further digested with NdeI (recognition site at bp 62 of the outside end). This digest was run on a 5% acrylamide gel, and a duplex fragment of approximately 128 bp was purified. The purified fragment was heated to 100°C for 2 min and quick cooled to convert the top and bottom strands into identical, self-complementary 128 nt hairpins. The DNA was then 5′ end labeled. A small proportion of the duplex DNA isolated from the polyacrylamide gel ($<$ 5%) included molecules in which only one of the two transposon strands had been ligated, giving rise to 65 and 63 nt strands after the heat treatment. As shown in Figure 5A, these species correspond to Tn-B and Tn-T, respectively.

The dG and ddG substrates (Figure 6A) were constructed by annealing an 88 nt nontransferred strand oligo comprised of bp 1–84 of the outside end and 4 nt (5′ GGCC 3′) of flanking DNA, to either (i) an 88 nt transferred strand oligo with its 3′ terminus at bp 1 and its 5′ terminus at bp 88, or (ii) to an 87 nt transferred strand oligo with its 3′ terminus at nt 2 and its 5′ terminus at nt 88 of the outside end. Duplex substrates were isolated from a 5% native polyacrylamide gel, and the latter was mixed with ddGTP and M-MuLV reverse transcriptase (NEBL) in RT buffer to incorporate ddG at nt 1. Incorporation of ddG was approximately 90% as determined by electrophoresis on an 8% denaturing gel.

Proteins

Transposase proteins were purified essentially as described in Chalmers and Kleckner (1994). Transposase concentrations were measured by the BCA assay (Pierce). IHF was purified and kindly provided by G. Chaconas.

Reaction Conditions

Standard reaction conditions for formation of synaptic complexes were as described in Sakai et al. (1995). ^{32}P-labeled substrate DNA ($\sim$100,000 cpm per 1$\times$ volume reaction) was first mixed with reaction buffer and then IHF. After 30 min transposase was added and incubation was continued for an additional 2 hr. To initiate the chemical steps in transposition, $MgCl_2$ was added to a final concentration of 5 mM. All incubations were at 20°C. Samples were analyzed by gel electrophoresis as described in Sambrook et al. (1989).

Quantification

Gels were dried down on Whatman 3MM paper at 80°C for 40 min and species visualized by phosphorimaging using a Molecular Dynamics Phosphoimager. The intensities of the various DNA species were determined using ImagQuant software. The 20% denaturing gel (Figure 6) was not dried down but frozen at −80°C and immediately exposed to film for up to 1 hr.

Kinetic Analysis of Cleavage

Values for Tn-B, HP, and FD-T were divided by the amount of starting substrate, URS T+B, at t = 0 and multiplied by 100 to give a percent of the total substrate converted. Since URS contains two end labels and each of the above products contains only one, URS values were divided by two. The labeling efficiency at the two 5′ ends of the substrate did not differ significantly ($<$5%), thus no additional corrections were necessary.

Levels of the cleaved end (CE) and nicked (N) forms present at each time point were calculated from the levels of relevant species as observed in denaturing gels using the following equations: (1) CE = [(FD-T) − HP] and (2) N = [(Tn-B) − CE]. Lifespans for the two intermediate forms, N and HP, were determined in two steps. First, the areas under the two corresponding noncumulative curves were determined (from Figure 2B and E). Second, those areas were divided by the total number of molecules that have gone through the reaction through the relevant point. For the wild-type reaction, which has essentially reached its final yield of $\sim$30% of the initial substrate converted during the reaction time examined, this value was taken as the value of Tn-B at the final time point. For the PS167 reaction, in contrast, only the nicking step of the reaction was completed to its final level in the period of the assay. Thus, total molecules were given by Tn-B at the final time point for calculating the lifespan of the N form; for the HP form, however, total molecules were taken to be the fraction not present in N or CE forms out of the total (N+CE+HP) at the final time point. The lifespans of N and HP intermediates calculated in this way were also used to determine cumulative curves for the corresponding N and HP stages. A cumulative curve describes, for each stage, how many molecules have achieved that stage as a function of time. The cumulative curve for the final cleavage product, the CE form, is given directly by the level of that form as a function of time. For any intermediate form, the corresponding cumulative curve is obtained through appropriate integration of the noncumulative curve as described in Padmore et al. (1991).

DNA Hairpin Purification and Enzymatic and Chemical Treatments

Hairpin was eluted from a 5% denaturing gel by soaking gel slices in 1$\times$ TE buffer for 16 hr at 37°C. Eluates were pooled and the DNA concentrated by ultrafiltration using a BioMax-10K NMWL membrane (Millipore). Hairpin ($\sim$300,000 cpm) was further purified by two rounds of centrifugation through Biospin 6 columns equilibrated in 1$\times$ DNA buffer. Hairpin ($\sim$20,000 cpm per reaction) was treated with P1 (Pharmacia) or mung bean nuclease (NEBL) for 1 hr at 20°C in 100 mM Tris-HCl (pH 7.5), 10 mM $ZnCl_2$. Reactions were terminated by phenol extraction. Chemical modification of hairpin ($\sim$100,000 cpm) with DMS and cleavage was performed as described in Sambrook et al. (1989).

Two-Dimensional Gel Analysis of Cleavage Reaction

A cleavage reaction with the standard substrate was terminated after 40 min by phenol extraction. DNA was precipitated with ethanol and run on a 5% native gel as described previously. The lane containing the DNA was cut out and soaked in 30% formamide, 8 M urea, 1$\times$ TBE for 45 min at room temperature. The gel slice was then placed between glass plates on top of an 8% polyacrylamide gel (16 cm $\times$ 14 cm $\times$ 0.08 cm) containing 8 M urea, 1$\times$ TBE, and electrophoresis in the second dimension was carried out for 2 hr at 100 V.

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