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The two single-strand cleavages at each end of Tn*10* occur in a specific order during transposition

(nicking/nonreplicative transposon)

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ABSTRACT **During Tn*10* transposition, the element is excised from the donor site by double-strand cleavages at the two transposon ends. Double-strand cleavage is a central step in the nonreplicative transposition reaction of many transposons in both prokaryotes and eukaryotes. Evidence is presented to show that the Tn*10* double-strand cut is made by an ordered, sequential cleavage of the two strands. The transferred strand is cut first, and then the nontransferred strand is cleaved. The single-strand nicked intermediate is seen to accumulate when Mn^{2+} is substituted for Mg^{2+} in the reaction or when certain mutant transposases are used. The fact that the transferred strand is cleaved before the nontransferred strand implies that the order of strand cleavages is not the determining factor that precludes a replicative mechanism of transposition.**

A fundamental difference between replicative and nonreplicative transposons is the type of cleavages that occur at the donor cleavage step (Fig. 1).

For replicative transposons such as phage Mu, only one strand of the donor DNA is cut at each end of the element and subsequently transferred to target sequences; the other, nontransferred strand is left uncut. Since the nontransferred strand at each end remains linked to flanking donor DNA, transposon sequences can be duplicated via DNA replication primed by the 3′-OH of flanking target sequences, forming a structure called a cointegrate (1).

For nonreplicative transposons, such as Tn*10* and Tn*7*, both strands of the donor DNA are cut at each end, resulting in complete separation of transposon sequences from flanking donor DNA (2–4). In this mode, transposition occurs without replication of transposon sequences as an intrinsic part of the reaction mechanism. Excision by double-strand breaks precludes cointegrate formation in these cases.

During Tn*10* transposition, transposase recognizes specific sequences at the two ends of the element and assembles a synaptic complex within which the chemical steps occur (refs. 5 and 6 and see below). Donor cleavage occurs by a pair of flush double-strand breaks at bp 1, and then the transferred strand (presenting the 3′-OH) at each end is joined to the target DNA (7).

Since donor cleavage is a central aspect of the nonreplicative transposition reaction, we have performed an analysis of how double-strand breaks occur during excision of Tn*10*. We report below kinetic experiments showing that the two DNA strands at each end of Tn*10* are cleaved sequentially. The transferred strand is always the first strand to be cleaved, producing a nicked intermediate. To further prove the existence of a nicked intermediate, we have sought and identified alterations of the reaction that accentuate the level of nicking of the transferred strand.

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MATERIALS AND METHODS

Transposition Reaction and Nick Detection Assay. Insertion sequence (IS) *10* transposase was purified as described (8), except that mutant screening used a minipurification (see below). The DNA substrate was obtained by *Sal* I/*Bgl* II digestion of plasmid pOH59 (6, 9), and it was end labeled with avian myelobastosis virus reverse transcriptase using [α-^{32}P]dATP (NEN; 6000 Ci/mmol; 1 Ci = 37 GBq). Reaction conditions and gel shift assay have been described (6).

For nick detection, the full-length fragment band from a nondenaturing gel (see Fig. 2*A*) was soaked in 400 μl of 0.5% SDS/0.3 M sodium acetate for >8 h at room temperature. The DNA was obtained free of acrylamide by phenol extraction and ethanol precipitation and analyzed in an 8% sequencing gel.

Screening for "Hypernicking" Mutants. Plasmid pNK2853 (7) contains the gene encoding IS*10* transposase under T7 promoter control. Plasmid was mutagenized with hydroxylamine (10) and used to transform NK8034 (λNK1275) (11). Resulting colonies were screened for papillation phenotypes (12). Colonies exhibiting 1–10% of the wild-type (pNK2853) papillation level were chosen for further analysis. For each of 140 such colonies, DNA was extracted and used to transform BL21(DE3)/pLysS (13), and transposase protein was prepared by minipurification. Each such preparation was assayed by gel retardation (6). Mutants that were able to form a synaptic complex but showed low levels of double-strand cleavage complexes were then screened for hypernicking phenotypes by the nick detection assay (above).

Transposase Minipurification. *Escherichia coli* BL21(DE3)/pLysS (13) containing plasmid pNK2853 (or a mutant derivative) was grown in Luria–Bertani broth at 37°C to an OD_{600} = 0.5–0.7. After a 2-h induction with 1 mM isopropyl β-D-thiogalactoside, 30 ml of the culture was centrifuged and resuspended in 0.5 ml of buffer A (25 mM Tes, pH 7.5/1 mM EDTA/10 mM dithiothreitol). Cells were frozen in dry ice and stored at −70°C. Frozen cells were thawed to 4°C and adjusted to 2 mM phenylmethylsulfonyl fluoride and 10% Triton X-100. After 30 min on ice, the extract was centrifuged 15 min in an Eppendorf centrifuge, washed with buffer A, and resuspended in 0.5 ml of buffer A. The solution was sonicated three times at 40 W for 20 sec and centrifuged for 10 min; the resulting supernatant was made 0.25 mM in ammonium sulfate. After overnight incubation at 4°C, the protein was collected by a 15-min centrifugation and resuspended in 20–50 μl of buffer A. An equal volume of buffer A containing 100 mM Triton X-100 and 4 M NaCl was added, and the tube was incubated on ice for 30 min and assayed immediately or stored at −70°C for further use. For the *in vitro* transposition reaction, this protein solution was typically diluted 1:500 in buffer contain-

Abbreviations: PEC, paired-ends complex; SEB, single-end break; DEB, double-end break. IHF, integration host factor; IS, insertion sequence.

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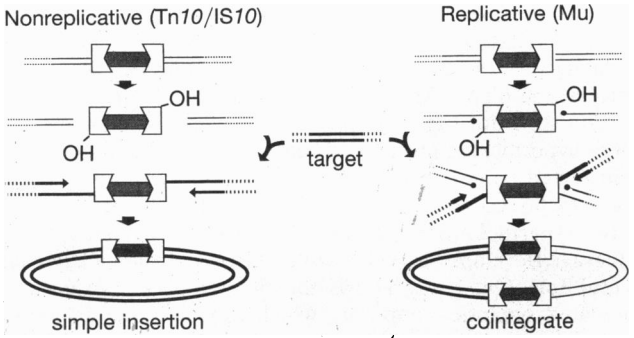

FIG. 1. Comparison of replicative and nonreplicative transposition. Both types of transposition expose the two 3′-OH transposon ends that subsequently join to target DNA in the strand transfer reaction. For nonreplicative transposition, cleavage of the two DNA strands at the two ends separates the element from flanking donor sequences. For replicative transposition, nicking at both transposon ends mantains the connection to flanking donor DNA and permits subsequent duplication of transposon sequences to form a cointegrate.

ing 10 mM Tris (pH 7.5), 50% (vol/vol) glycerol, 10 mM dithiothreitol, 1 mM EDTA, 2 M NaCl, and 1% (wt/vol) bovine serum albumin.

RESULTS

Detection of Nicks in the Wild-Type Transposition Reaction. The entire Tn*10* transposition reaction can be reconstituted *in vitro* on short linear fragments bearing transposon end sequences (6). In such a reaction, IS*10* transposase organizes a protein–DNA synaptic complex containing a pair of transposon end fragments; integration host factor (IHF) is an essential accessory factor for this step. In the presence of Mg^{2+}, these complexes undergo double-strand cleavage at both transposon ends. If target DNA is added to the reaction, strand transfer occurs.

We have used this reaction to monitor the formation of nicks during the transposition reaction. Substrate DNA (Fig. 2*A*) was incubated overnight in the presence of transposase and IHF and in the absence of divalent metal ion. Mg^{2+} was then added to the reaction mixture ($t = 0$), and at various times thereafter, portions of the reaction were withdrawn, treated with SDS to stop the reaction, subjected to phenol extraction, and assayed for the nature of DNA species present.

DNA was first analyzed in double-stranded form by electrophoresis in a nondenaturing polyacrylamide gel (Fig. 2*A*). Full-length duplex fragment (which might be intact or nicked) was separated from the two smaller species generated by double-stranded cleavage at the transposon end. Quantitation of these species shows that double-strand breaks accumulate throughout the time course until a maximum level is reached at 8 h (Fig. 2*C*). Cleavage has gone to completion in this reaction; the proportion of substrate fragments cleaved (33%) corresponds precisely to the proportion of substrate fragments present in synaptic complexes at $t = 0$ in the same reaction as assayed by gel retardation (34%; see below).

The occurrence of nicks was assessed by eluting the full-length duplexes from the nondenaturing gel and examining the component single strands by electrophoresis in a denaturing polyacrylamide gel (Fig. 2*B*). Unnicked single strands are 136 nt in length; nicks at nt 1 on the transferred strand should produce a 49-nt radiolabeled species; nicks at nt 1 on the nontransferred strand should produce an 87-nt radiolabeled species.

Nicks are observed specifically at nt 1 on the transferred strand at time points between 10 min and 8 h after addition of Mg^{2+}. In no case were nicks detected on the nontransferred strand. Moreover, nicks on the transferred strand show the kinetics of a reaction intermediate, rising at early times, reaching a maximum at 40 min, and fading away at later times.

Nicks persist for a significant period of time during the reaction. The average lifespan of the nicked species in these reactions is 25 min. This lifespan is given by the area under the corresponding curve in Fig. 2*C* (in units of relative level of

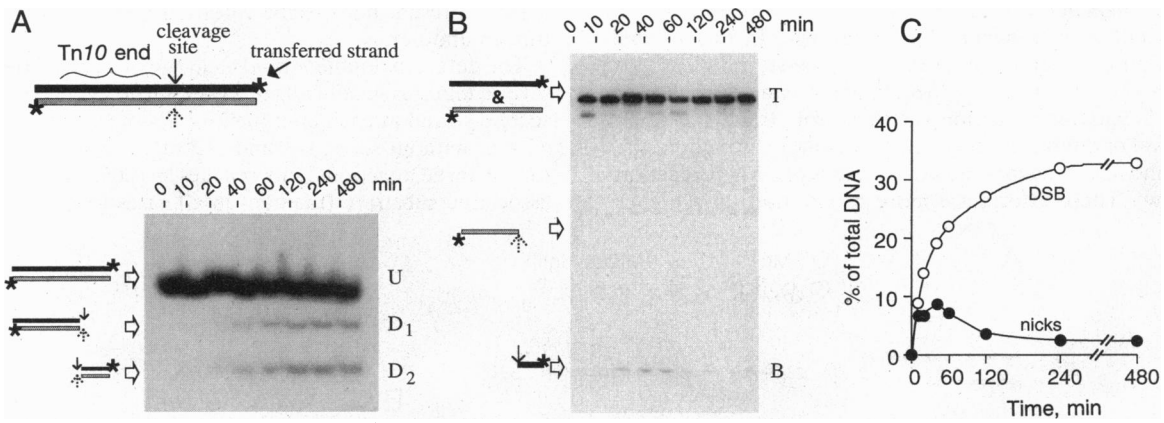

FIG. 2. Detection of nicks in the standard reaction. (*A*) The substrate was a labeled 136-bp fragment that contains bp 1–81 of the outside Tn*10* end plus 49 bp of flanking DNA adjacent to bp 1 plus an additional 6 bp beyond bp 81. An asterisk indicates the position of the radioactive label. The fragment was incubated overnight with transposase and IHF to allow synaptic complex formation. Five millimolar $MgCl_2$ was then added; reactions were stopped at different times thereafter by addition of 0.5% SDS, proteinase K incubation (20 μg/ml for 30 min), and phenol extraction. The products of the reaction, taken at different times after the addition of Mg^{2+}, were separated in a 5% nondenaturing polyacrylamide gel. Uncleaved DNA runs at the top of the gel (136 bp = U) while the two faster migrating bands are double-strand cleavage products (87 bp = D_1, 49 bp = D_2). (*B*) Uncleaved DNA (U) from the gel in *A* was eluted and rerun in a denaturing gel. Nicks on the transferred strand are observed as a 49-nt band (bottom band = B). Nicks on the nontransferred strand (expected in the middle area of the gel) are not observed. The top band (T) contains unnicked DNA strands; the observed shadow band is presumably due to incomplete denaturation. (*C*) Graphic representation of data from *A* and *B*. Radioactivity was quantified using a Fuji Bas2000/Phosphorescence imager. The absolute number of radioactive counts was corrected for the differing efficiencies with which the labels at the two substrate ends were introduced in the labeling reaction. In this experiment the transferred strand was labeled 1.3 times more efficiently than the nontransferred strand. To calculate relative molar amounts, counts obtained from the transferred strand were divided by 1.3, and counts from duplexes were divided by 2.3. ○, percentage of duplexes that have undergone double-strand break (DSB), determined at each time point from the gel in *A* using the formula $\{(D_2/1.3)/[(D_1 + D_2 + U)/2.3]\} \times 100$. ●, percentage of nicked duplexes (nicks) relative to the DNA that did not undergo double-strand break (U in the gel in *A*), calculated as $\{(B/1.3)/[(T + B)/2.3]\} \times 100$.

DNA molecules × time) divided by the relative level of DNA molecules that eventually undergo at least one such nick (34% in these reactions).

Identification of Hypernicking Transposase Mutants. Knowing that a nick on the transferred strand is a normal intermediate in the reaction pathway, we thought it possible to screen for mutations that confer a specific block after this nick is made. Such mutations would accentuate the level of nicking on the transferred strand when assayed by the nick detection assay. We first performed an *in vivo* screen for transposition-defective mutants using a previously described papillation assay in which transposition results in turn-on of a *lacZ* gene (14). One hundred forty mutants that exhibited between 1% and 10% the transposition activity of the wild-type transposase construct were identified from hydroxylamine-mutagenized DNA. For each of these candidates, a small-scale transposase preparation was made and assayed *in vitro* for competence in synaptic complex formation and cleavage at the transposon ends. Four of these candidates were still able to form a synaptic complex containing a pair of transposon ends but were defective in double-strand cleavage. Analysis in the nicking assay revealed that three of these four showed higher levels of nicking than wild type.

The three hypernicking mutants are AT162, EK263, and MI289. They behave identically in both the nicking assay and in other assays reported below. In all three mutant cases, nicks appear at about the normal time in the reaction but accumulate to high levels. Only nicks on the transferred strand are observed (Fig. 3).

The three mutants identified here to have a hypernicking phenotype have been isolated previously in a screen for mutants that retain the wild-type capacity to induce host SOS functions in the presence of transposon ends (SOS^+ $Tnsp^-$; ref. 13). These three are among a subset of SOS^+ $Tnsp^-$ mutants whose transposition defects were strong but not absolute. The identification of hypernicking mutants in this screen is explained by the finding that all three mutants form substantial levels of double-strand breaks on one of the transposon ends *in vitro* (see below).

Mn^{2+} Confers a Hypernicking Phenotype. In the *in vitro* transposition assay, optimal levels of double-strand cleavages are obtained with 5 mM Mg^{2+} (8, 9). Mn^{2+} can substitute for Mg^{2+} in the cleavage reaction (and also for strand transfer), but the level of double-strand cleavage is lower throughout the reaction and never reaches the same level as in a Mg^{2+} reaction (see below). These effects are more pronounced at a higher concentration of Mn^{2+} (5 mM) than at a lower concentration (0.2 mM) (data not shown).

Analysis of the transposition reaction by the nick detection assay at the higher Mn^{2+} concentration reveals that substitution of Mn^{2+} for Mg^{2+} confers a phenotype analogous to the three hypernicking mutations. Nicks are observed only on the transferred strand and occur in a higher fraction of synaptic complex-bound ends than in reactions with Mg^{2+} (Fig. 3).

In Hypernicking Situations, the Block to Second-Strand Cleavage Develops Several Minutes After Addition of Divalent Metal Ion. Our assay allows the detection of transposase-mediated synaptic complexes, which can be observed as retarded bands that appear in acrylamide gels when the loaded samples contain DNA and proteins in their native state (6) (Fig. 4). The presence of transposase results in the formation of a paired-end complex (PEC) that contains two transposon end fragments. Essentially, all the substrate not present in complexes is shifted in the gel due to efficient IHF binding. Upon addition of divalent metal ion, double-strand cleavages can be observed as changes in the gel mobility of the synaptic complex due to release of flanking DNA segment(s). In the wild-type reaction, double-strand cleavages occur successively at one end and then the other, as seen by the conversion of PEC into single-end break (SEB) complex and then double-end break (DEB) complex (6) (Fig. 4*A*).

Under hypernicking conditions, in the presence of Mn^{2+} or with MI289 transposase, the kinetics of cleaved complex progression are rather unusual (Fig. 4 *B* and *C*). For the first several minutes of the reaction, cleavage occurs relatively normally: 10 min after addition of divalent metal ion, 30–38% of complexes are in the SEB form and 7% are in the DEB form (the corresponding values for the wild-type reaction are 40% for SEB and 10% for DEB). Abruptly, however, the occurrence of double-strand cleavages slows dramatically, and after 4 h, SEB and DEB complexes make up only ≈50% and ≈12% of total synaptic complexes, respectively (in the wild-type reaction, almost 100% of complexes are in the DEB form). Minor differences between the two hypernicking cases might easily represent nonspecific variation from one reaction mixture to another.

The defect in double-strand cleavage observed after the first several minutes specifically represents a defect in nicking of the second strand at each end: the kinetics of first-strand nicking, with or without second-strand nicking, is essentially identical for all three reactions and reaches ≈100% of the complex-associated substrate fragment in all cases (Fig. 5).

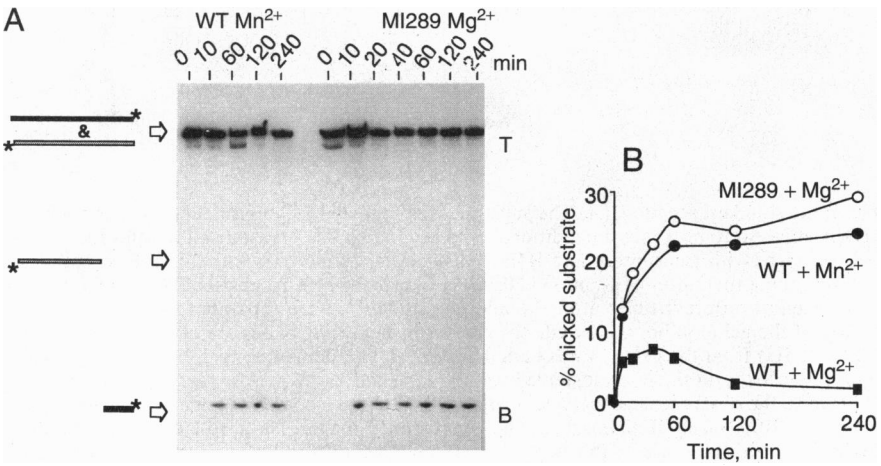

FIG. 3. Hypernicking phenotype showed by mutant MI289 or by the presence of Mn^{2+} in the reaction with wild-type transposase. (*A*) Following the same procedure as in Fig. 2, nicks were detected in reactions using mutant MI289 instead of wild-type (WT) transposase or using 5 mM Mn^{2+} instead of Mg^{2+} with wild-type transposase. (*B*) Nicks were quantified as described in Fig. 2. ■, wild-type transposase + 5 mM $MgCl_2$; ●, wild-type transposase + 5 mM $MnCl_2$; ○, MI289 transposase + 5 mM $MgCl_2$.

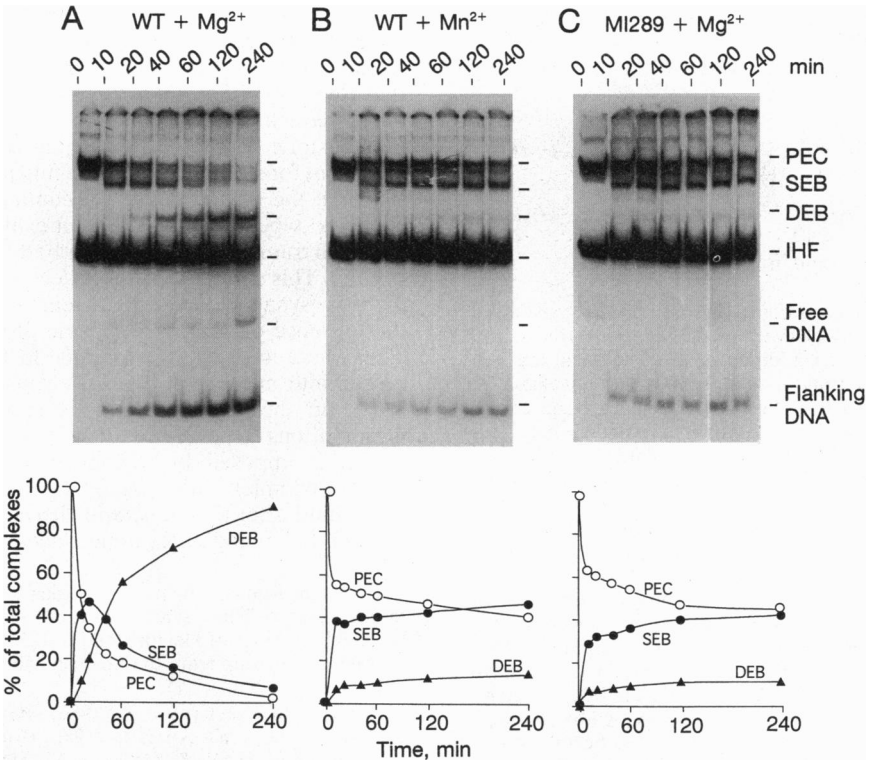

FIG. 4. Kinetics of cleaved complex progression in the standard reaction (*A*) and two hypernicking situations (*B* and *C*). The same time courses shown in Figs. 2 and 3 were used to observe the formation of various complex species. The reaction was loaded onto a 5% acrylamide native gel at different times after addition of Mg^{2+} or Mn^{2+}, without the protein extraction step. ○, PEC; ●, SEB complex; ▲, DEB complex; IHF, substrate bound to IHF. Flanking DNA gets released from the complex after end cleavage. For the graphical representation, the percentage of each complex at each time point was calculated relative to the total amount of complexes (PEC + SEB + DEB = 100%). Because of the differing efficiencies with which the labels at the two substrate ends were introduced in the labeling reaction, counts were divided by 4.6 (PEC), by 3.3 (SEB), or by 2 (DEB) to obtain relative molar amounts of each complex species. Radioactive counts present in the wells were omitted in the calculations; in all cases they represented <10% of the counts present in complexes.

DISCUSSION

Transposition Involves Sequential Nicking on the Transferred and Nontransferred Strand at each Transposon End. We report here that the two single strands at each end of Tn*10* are cleaved in a specific order during transposition. In the course of an *in vitro* reaction in the presence of Mg^{2+}, we detect nicks on the transferred strand, and these nicks exhibit the kinetics expected for a reaction intermediate. Nicks on the nontransferred strand are never observed, suggesting that there is an obligatory order in the two single-strand cleavages at each end—i.e., that the transferred strand is always cleaved before the nontransferred strand.

The idea that Tn*10* transposition occurs via a specific nicked intermediate is reinforced by the observation of alterations in the reaction that give rise to accumulation of nicks on the transferred strand. A hypernicking phenotype is conferred by substitution of Mn^{2+} for Mg^{2+} in an otherwise wild-type reaction. In addition, a screen of transposition-defective mutants has identified point mutations in IS*10* transposase (AT162, EK263, and MI289) that confer a hypernicking phenotype; in all three mutants, nicks are accumulated on the transferred strand.

In both hypernicking situations, the kinetics of double-strand cleavage are biphasic. For the first 10 min, the level of double-strand breaks rises normally and the percentages of complexes cleaved at one or both ends is the same as in a wild-type reaction. After that time, the level of double-strand cleavage plateaus, with the level of SEB and DEB complexes remaining essentially constant thereafter. In contrast, first-strand nicking occurs normally throughout the reaction. We interpret this pattern to mean that there is a change in the reaction several minutes after addition of divalent metal ion and that this change, which is probably a conformational change of transposase within the synaptic complex, specifically prevents cleavage of the second strand. Since first-strand nicking continues, all of the substrate fragment associated with complexes eventually becomes nicked.

It should be noted that the observed pattern cannot be explained by the existence of a block between double-strand cleavage at the first end and double-strand cleavage at the second end; such a block would have resulted in continued formation of SEB complexes to a high level rather than the coordinate cessation of double-strand breakage at both first and second ends, as is observed.

Mechanistic Basis for Ordered Nicking. A number of different scenarios could account for the occurrence of the two strand nicks in what appears to be an obligatory specific order. Among these, we currently favor the view that each end of the element is cleaved on both strands via a single active site located within a single monomer of transposase. Such a mechanism would directly and necessarily ensure that the strands are cleaved sequentially and would almost certainly imply that the cleavages would occur in a specific order. Such a model is also consistent with additional observations suggesting that one monomer of transposase is able to cleave both DNA strands at a single end (S.B. and N.K., unpublished results).

In alternative models, the specific order of strand cleavages could be due to a functional interdependency between the two cleavages, with cleavage of the first strand required for cleavage of the second strand. Another possibility is that the specific order could be entirely a kinetic effect in which the transferred strand is cleaved appreciably faster than the independently

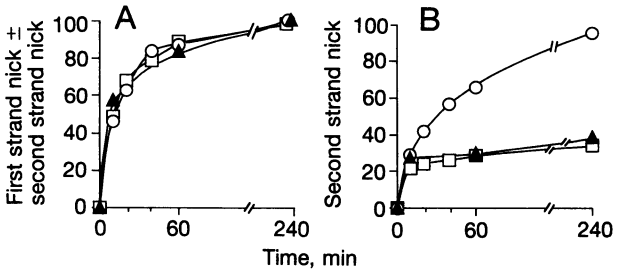

FIG. 5. In hypernicking conditions, cleavage of the transferred strand is not affected, whereas cleavage of the nontransferred strand is blocked several minutes after addition of divalent metal ion. (*A*) First strand nick ± second strand nick represents the percentage of complex-associated duplexes that have undergone cleavage of the transferred strand, regardless of whether they have undergone cleavage of the second strand or not. They were calculated by adding the observed nicks and double-strand breaks at a particular time point. 100% corresponds to all the DNA forming synaptic complexes—that is, 34% of total DNA in reactions performed with wild-type transposase and 42% of total DNA in reactions with MI289 transposase. Only DNA included in synaptic complexes is susceptible to nicking, as has been confirmed for the Mn^{2+} reaction by analysis of nicks in all relevant retardation gel species present after 4 h. Nicks were absent from free DNA and IHF-bound DNA (<0.5%) and are rare in DNA present in the wells (<5%); for PECs and SEBs, in contrast, all uncleaved fragment was nicked (100% and 50% of total fragment, respectively). ○, Wild-type transposase + Mg^{2+}; ▲, wild-type transposase + Mn^{2+}; □, MI289 transposase + Mg^{2+}. (*B*) Second strand nick represents the percentage of complex-associated duplexes that have undergone double strand breaks, calculated as % double end breaks (DEB) + ½% single end breaks (SEB).

cleaved nontransferred strand. This latter model does not seem very likely because it predicts that we should have found mutations that block cleavage of only the nontransferred strand as well as mutations that block cleavage of only the transferred strand. It is possible, however, that such mutations occur but were not recovered in this screen.

What Precludes Replicative Transposition During Tn*10* Transposition? It is interesting to note that the observed order of strand cleavage in Tn*10* would be compatible with a replicative mode of transposition. Interruption of the cleavage process after the first nick would generate 3′-OH termini that are chemically proficient for strand transfer as during Mu transposition. Had the nontransferred strand been cleaved before the transferred strand, Tn*10*-promoted cointegrate formation would have been precluded as an intrinsic consequence of the reaction order: the 3′-OH termini required for strand transfer would have been generated in the same step that cleaved the second strand.

We have investigated in a preliminary way the possibility that synaptic complexes containing nicked termini might be proficient for strand transfer. We find no evidence for any such reaction: the complexes that accumulate in a Mn^{2+} reaction with wild-type transposase do not exhibit any detectable level of strand transfer at either one or both ends (S.B., unpublished results). This defect does not reflect a block to strand transfer *per se*, as synaptic complexes assembled on precleaved ends in the presence of Mn^{2+} are capable of joining to target DNA. Thus, there must be an additional factor(s) that prevents the nicked intermediate in Tn*10* transposition from being transferred to the target before the second strand is cleaved. Observations to be described elsewhere suggest that the constraint is imposed by a conformational block within the synaptic complex, which prevents stable interaction with target DNA until after double-strand cleavage has occurred at both ends (J. Sakai and N.K., unpublished results).

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