Identification and Characterization of the Linear IS3 Molecules Generated by Staggered Breaks*

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Insertion sequence IS3 encodes two, out-of-phase, overlapping open reading frames, orfA and orfB. The OrfAB transframe protein that is IS3 transposase is produced by a translational frameshifting between orfA and orfB. Efficient production of the IS3 transposase in the cells harboring the IS3-carrying plasmid has been shown to generate miniplasmids as well as characteristic minicircles, called IS3 circles, consisting of the entire IS3 sequence and one of the 3-base pair sequences flanking IS3 in the parental plasmid. Here, we show that the IS3 transposase also generates the linear molecules of IS3 with 3-nucleotide overhangs at the 5′-ends. The nucleotide sequences of the overhangs are the same as those flanking IS3 in the parental plasmid, suggesting that the linear IS3 molecules are generated from the parental plasmid DNA by staggered double strand breaks at the end regions of IS3. The linear IS3 molecules are likely to be the early intermediates in the transposition reaction, which proceeds in a non-replicative manner.

Transposable elements are characterized by their ability to transpose, and many of such elements have been identified so far in plasmids and chromosomes of a wide variety of organisms. IS3 (1258 bp in length) is an insertion element present in the Escherichia coli chromosome and in plasmid F (Malamy et al., 1972; Hu et al., 1975; Deonier et al., 1979; Timmerman and Tu, 1985; Umeda and Ohtsubo, 1989). IS3 does not mediate cointegration and is thus supposed to transpose in a non-replicative manner (Sekine et al., 1994). This element has imperfect inverted repeats (IRL and IRR) of 39 bp at its terminal regions and encodes two open reading frames, orfA and orfB, which are in phase 0 and 1, respectively, and overlap each other (Timmerman and Tu, 1985; Fayet et al., 1990; Sekine and Ohtsubo, 1991). In addition to the OrfA and OrfB proteins, which are produced from each of the two orfs, a transframe protein (the OrfAB protein) that is IS3 transposase is produced by a translational frameshifting at the A₆G motif present in the overlapping region between the two orfs (Sekine et al., 1994). An IS3 mutant with a single guanine insertion in the A₆G motif to give A₆G₂ produces the transposase protein without frameshifting. This mutant causes a deletion of a sequence adjacent to IS3 in a plasmid to produce miniplasmids and generation of characteristic minicircles, called IS3 circles, which consist of the entire IS3 sequence and a 3-bp sequence intervening between the IS3 ends (Sekine et al., 1994). The orfB frame of IS3 codes for a polypeptide segment showing homology with a conserved amino acid sequence motif found in retrovirus and retrotransposon integrases (Fayet et al., 1990; Khan et al., 1991), while the orfA frame codes for a polypeptide segment with the α-helix-turn-α helix motif, which may be involved in recognition of the IS3 end regions (Prère et al., 1990; Sekine and Ohtsubo, 1993). A group of IS elements that are structurally related to IS3 have been isolated from diverse bacterial genera. These elements, called the IS3 family (Schwartz et al., 1988), code for two orfs in phase 0 and 1, respectively (Fayet et al., 1990; Sekine and Ohtsubo, 1991), and the predicted amino acid sequences encoded by the downstream orf are similar to one another and have homology with the motif in retrovirus/retnransposon integrases (Fayet et al., 1990; Khan et al., 1991). In addition to IS3, two other members of the family produce transposases by framing between the two orfs (Vögeler et al., 1991; Polard et al., 1992).

Here, we report that IS3 transposase generates linear IS3 molecules in addition to miniplasmids and IS3 circles. The molecules have 5′-overhangs, suggesting that they are generated from the parental plasmid DNA carrying IS3 directly by staggered breaks. We point out that the transposition reaction in IS3 is similar to the transposition reaction in other transposons, such as Tn10 and Tn7, which occurs by a non-replicative mechanism (Morisato and Kleckner, 1984; Benjamin and Kleckner, 1989; Bainton et al., 1991), and even more to the integration reaction in retroviruses, which generate linear DNA molecules as well as characteristic circles with long terminal repeat sequences.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Bacterial strains used were E. coli K12 derivatives MV1184 (Vieira and Messing, 1987) and YK1100 (Wada et al., 1988). Plasmids p5EK183 and p5EK1831 were used as pUC118 derivatives and carry wild-type IS3 and an IS3 mutant, IS3-1, respectively (Sekine et al., 1994). IS3-1 contains a guanine insertion in the A₆G motif at nt 328–332 by the coordinates given to the IS3 sequence (Timmerman and Tu, 1985), leading to in-frame alignment of orfA and orfB. Plasmid p5EK1832 belong to type I miniplasmids derived from p5EK1831 and is deleted for the region (1018 bp) extending from the end of IRR to a site within the IG region in p5EK1831 (Sekine et al., 1994).

Media—Culture media used were L broth and L rich broth (Yoshikawa et al., 1987). L-agar plates contained 1.5% (w/v) agar (Eiken) in L broth. Antibiotics were added in L-agar plates, when necessary, at the concentration of 100 μg of ampicillin (Wako) ml⁻¹ and 30 μg of chloramphenicol (Sigma) ml⁻¹.

Enzymes, Reagents, and Chemicals—Enzymes used were T4 polynucleotide kinase, exonuclease III, restriction endonuclease MluI, bacterial alkaline phosphatase, and DNA polymerase I (Klenow fragment) (Takara); λ exonuclease (Life Technologies, Inc.); restriction endonucleases, BsmI and Ncol (New England Biolabs); and modified T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.).
Reagents and chemicals used were [γ-32P]ATP (222 T BPM/mmol) and [α-32P]dNTP (110 TBq/mmol) (Amersham Corp.); dNTPs and agarose (Takara); polyacrylamide (Wako).

DNA Preparation—DNA was prepared from the E. coli cells grown in L rich broth. The alkaline lysis method (Sambrook et al., 1989) was used to prepare plasmid DNA for cloning and nucleotide sequencing. Small scale preparation of DNA for detection of the linear IS3 molecules was performed under the neutral condition as described by He et al. (1990).

Large scale preparation of the linear IS3 molecules was carried out as follows. Strain MV1184 harboring pSEK1831 or pSEK1832 was grown in 500 ml of L rich broth overnight at 37 °C. The cells were collected and lysed by the method of Clewell and Helinski (1970). The DNA was separated by CsCl/ethidium bromide equilibrium density centrifugation (Sambrook et al., 1989), and the upper band DNA containing linear DNA molecules and nicked circular plasmid DNA were collected. The DNA preparation was then separated by electrophoresis in a 1.2% agarose gel, and the band of the linear IS3 molecules was cut out and eluted.

Oligonucleotides—Oligonucleotides were synthesized using a DNA synthesizer model 392 (Applied Biosystems) and were labeled, if necessary, at their 5'-ends using T4 polynucleotide kinase and [γ-32P]ATP.

Nucleotide Sequencing—Nucleotide sequences were determined by the dideoxynucleotide method (Sanger et al., 1977; Messing, 1983) using the Sequenase DNA sequencing kit (U.S. Biochemical Corp.) following the recommendations of the manufacturer.

Analysis of the Structure of the Ends of the Linear IS3 Molecules—The 5'-ends of the linear IS3 molecules were analyzed by primer extension as follows. The linear IS3 molecules (0.02 pmol) were isolated from strain MV1184 harboring pSEK1831 as described above, and the 32P-labeled oligonucleotides (0.02 pmol) were annealed in the buffer containing 7 mM Tris HCl (pH 7.5), 0.1 mM EDTA, 20 mM NaCl, and 7 mM MgCl2. The reaction was carried out in the presence of dNTPs (0.2 mM each) and 0.1 unit of DNA polymerase I (Klenow fragment) for 40 min at 37 °C and stopped by adding formamide to a final 30% concentration (v/v). Primers used were L and R (see Table I). Size markers appearing as sequence ladders were prepared using the 32P-labeled L or R primer and the pSEK183 DNA as template. The DNA chains were separated on 8% polyacrylamide sequencing gels containing 7 M urea.

The 3'-ends of the linear IS3 molecules were analyzed as follows. The linear IS3 molecules were isolated from strain MV1184 harboring pSEK1831 as described above. In one reaction, the linear IS3 molecules (0.01 pmol) were digested with BsmI or NcoI, treated with bacterial alkaline phosphatase, and labeled with [γ-32P]ATP. In the other reaction, the linear IS3 molecules were treated with bacterial alkaline phosphatase, labeled with [γ-32P]ATP at their 5'-ends, and then digested with BsmI or NcoI. The samples so prepared were treated with alkaline-denatured and electrophoresed in 8% polyacrylamide sequencing gels containing 7 M urea. Size markers appearing as sequence ladders were prepared using the pSEK183 DNA as template and the 32P-labeled BsmI or NcoI primer (see Table I).

Determination of the Nucleotide Sequence of the Overhangs at the 5'- and 3'-Ends of the Linear IS3 Molecules—The linear IS3 molecules were isolated from strain MV1184 harboring pSEK1832, as described above. Seven µl of the buffer (27 mM Tris HCl, pH 7.5, 33 mM NaCl, 13 mM MgCl2, 6.7 mM dithiothreitol) containing the linear IS3 molecules (0.01 pmol) was mixed with 0.5 µl of [α-32P]dNTP in the presence or absence of 0.2 µM dNTP. A 1.6 unit of modified T7 DNA polymerase (Sequenase) was then added and incubated for 5 min at 37 °C. The samples were treated with BsmI or NcoI, heat-denatured, and then electrophoresed in 8% polyacrylamide sequencing gels containing 7 M urea. Size markers appearing as sequence ladders were prepared using the pSEK1832 DNA as template and the 32P-labeled BsmI or NcoI primer (see Table I).

RESULTS

Generation of the Linear IS3 Molecules

We have previously demonstrated that plasmid pSEK1831 carrying an IS3 mutant (IS3-1) with a guanine insertion in the A4G motif to give A4G2 generates major six types of small closed circular molecules (named I–VI in order of their sizes) (Fig. 1Aa, lane 1) due to the efficient production of IS3 transposase from the mutant without frameshifting (Sekine et al., 1994). Structural analyses of these molecules have revealed that type I–IV molecules are miniplasmids and type V molecules are minicircles lacking the region required for replication of the plasmid. These miniplasmids and minicircles are probably produced by deletion of a sequence adjacent to IS3. Most of the type VI molecules are IS3 circles, which consist of the entire IS3 sequence and a 3-bp sequence intervening between the IS3 ends (Sekine et al., 1994). All these molecules were identified in the DNA sample prepared under the alkaline condition from the cleared cell lysate. Here, we prepared the DNA sample under the neutral condition and found that the sample contained all types of molecules previously identified, in which, however, the band of the molecules corresponding to type V minicircles was denser than that in the sample prepared under the alkaline condition (Fig. 1Aa, lane 2). The DNA molecules migrated even faster than the IS3 circles (type VI molecules) and formed a distinct band in a polyacrylamide gel (Fig. 1Ba, lane 2; Fig. 1Bb, lane 1). When the DNA sample prepared under the neutral condition was treated with E. coli exonuclease III, such molecules disappeared, while the other molecules did not (Fig. 1Bb, lane 2). This shows that these molecules are linear DNA fragments with the 5' protruding or blunt ends that can be digested with E. coli exonuclease III. When the DNA sample was treated with λ exonuclease, the
linear molecules also disappeared (Fig. 1Ab, lane 3), showing that the linear DNA fragments have the 5'-ends with phosphate residues that can be digested with λ exonuclease.

The linear molecules are approximately 1.3 kb in length, as estimated from their electrophoretic mobility. This size is almost the same as that of the IS3 sequence (1,258 bp). When the DNA sample prepared under the neutral condition was digested with MluI, which cleaves the IS3 sequence at one site, there appeared two bands of DNA fragments, about 0.8 and 0.5 kb in length (see the bands indicated by open arrowheads in Fig. 1Bb, lane 3). Such DNA fragments were not contained in the DNA sample treated with exonuclease III, followed by digestion with MluI (see Fig. 1Bb, lane 4). The sizes of the two fragments are the expected ones of the MluI-digested IS3 sequence, which are 0.78 and 0.48 kb in length, suggesting strongly that the molecules identified above are the linear IS3 molecules. Note here that MluI digestion resulted in a shift of the position of the band corresponding to IS3 circles (type VI molecules) to the position where the linear IS3 molecules were originally present, due to conversion from the circular form to the linear form of the IS3 circles (Fig. 1Ab, lane 4; Fig. 1Bb, lanes 3 and 4).

The linear IS3 molecules as well as small circular molecules including IS3 circles were not detected in the DNA sample prepared under either the alkaline or neutral condition from the cleared lysate of cells harboring pSEK183 carrying wild origin.

**Structure of the Linear IS3 Molecules**

### Presence of Non-IS3 Sequence of 3 nt at the 5'-Ends—The linear IS3 molecules in the DNA sample prepared under the neutral condition from the cells harboring pSEK183 were purified by ultracentrifugation, followed by agarose gel electrophoresis (see "Materials and Methods"). The 5'-ends of the molecules were then analyzed by primer extension using oligonucleotide primers, L and R (Table I), which hybridize with the IS3 end regions, IRL and IRR, respectively, and prime DNA synthesis toward the outside of IS3 (Fig. 2A). The extension product from the L primer labeled with 32P at its 5'-end was 55 nt, which is 3 nt longer than the extension product terminating at the exact end of IRL of IS3 (Fig. 2Ba), whereas the extension product from the R primer labeled at its 5'-end was 55 nt, 3 nt longer than the extension product terminating at the exact end of IRR (Fig. 2Bb). These results show that the linear IS3 molecules have a 3-nt sequence at the 5'-ends of IS3.

### Presence of No Additional Nucleotides at the 3'-Ends—To determine the 3'-ends at IRL of the linear IS3 molecules, the molecules were digested with BsmI, which cleaves IS3 at one site, and the fragments containing IRL were isolated by polyacrylamide gel electrophoresis and labeled with 32P at their 5'-ends (see Fig. 3A, strategy 1). After denaturation and electrophoresis of the fragments in a sequencing gel, we detected two bands of single-stranded DNA fragments (Fig. 3Ba, lane 1). The small DNA fragment is supposed to correspond to the fragment, whose 3'-end is the IRL-proximal end of the linear IS3 molecule and whose 5'-end is labeled with 32P at the BsmI site (Fig. 3Aa), strategy 1. The large fragment is supposed to be the fragment with a 3-nt sequence attached to the 5'-end of IRL (Fig. 3Aa, strategy 1). To confirm this, the linear IS3 molecules were labeled with 32P at their 5'-ends and then digested with BsmI (Fig. 3Aa, strategy 1), and the DNA sample was denatured and electrophoresed in a sequencing gel. The large fragment was generated, but the small one was not (Fig. 3Bb, lane 2), confirming the assumption above. The small fragment was determined precisely by the size markers to be 105 nt long (Fig. 3Ba), indicating that the 3'-end at IRL of the linear IS3 molecules is the 3'-end of the IS3 sequence.

### To determine the 3'-ends at IRR of the linear IS3 molecules, the fragments containing IRR were isolated after digestion of the molecules with NcoI, which cleaves IS3 at one site, and labeled with 32P at their 5'-ends (see Fig. 3A, strategy 1). After denaturation of the sample and electrophoresis of the fragments in a sequencing gel, we detected two bands of single-stranded DNA fragments (Fig. 3Bb, lane 1). The large DNA fragment is supposed to correspond to the fragment, whose 3'-end is the IRR-proximal end of the linear IS3 molecules and whose 5'-end is labeled with 32P at the NcoI site. The small fragment is supposed to be the fragment with a 3-nt sequence attached to the 5'-end of IRR (Fig. 3Aa, strategy 1). To confirm this, the linear IS3 molecules were labeled with 32P at their 5'-ends and then digested with NcoI (Fig. 3Aa, strategy 1), and the DNA sample was denatured and electrophoresed in a sequencing gel. The small fragment was generated, but the large one was not (Fig. 3Bb, lane 2), confirming the assumption above. The small fragment was determined precisely by size markers to be 171 nt long (Fig. 3Bb), indicating that the 3'-end at IRR of the linear IS3 molecules is the 3'-end of the IS3
Determination of the Nucleotide Sequences of Overhangs at the 5'-Ends of the Linear IS3 Molecules

A, polyacrylamide gels (8%) showing the BsmI fragment having incorporated dNTP(s) into the 3'-ends at IRL of the linear IS3 molecules. B, polyacrylamide gels (8%) showing the NcoI fragment having incorporated dNTP(s) into the 3'-ends at IRR of the linear IS3 molecules. The DNA sample in each lane was obtained by incubation of modified T7 DNA polymerase (Sequenase) in the presence of dNTP(s) indicated. Sizes of the single-stranded DNA fragment in nt are indicated. Lanes marked M are the sequence ladders used as size markers, which were prepared using pSEK1832 as template and the BsmI primer (panel A) or NcoI primer (panel B). Nucleotide sequences of critical regions around IRL and IRR in pSEK1832 are indicated on the side of the gels together with coordinates to IS3.

The 3'-ends of IS3 molecules are generated by staggered breaks. The IS3 sequence and the overhanging sequences at the 5'-ends of IS3 are indicated by shaded and open thick lines, respectively. Filled boxes indicate the BsmI and NcoI primers (see Table I) used to prepare sequence ladders using pSEK1832 as template. Striped arrows indicate the direction of synthesis of DNA extended from each primer. Asterisks indicate the 5'-ends labeled with 32P. Sizes of the single-stranded DNA fragments are indicated in nt. B, polyacrylamide gels showing single-stranded DNA fragments generated upon denaturation of the linear IS3 molecule. Panel a, an 8% polyacrylamide gel showing DNA fragments generated upon BsmI digestion. Panel b, an 8% polyacrylamide gel showing DNA fragments generated upon NcoI digestion. Lane 1, the DNA sample obtained by restriction enzyme digestion followed by labeling with 32P as depicted in strategy I in A. Lane 2, the DNA sample obtained by labeling with 32P followed by restriction enzyme digestion as depicted in strategy II in A. Lanes marked M are the sequence ladders used as size markers, which were prepared using BsmI primer or NcoI primer. Sizes of the single-stranded DNA fragments are indicated in nt. The sizes of the DNA fragments with the 3-nt sequence attached to the 5'-ends are shown in parentheses, since they are calculated based on the results shown in Fig. 2. Nucleotide sequences of critical regions around IRL and IRR in pSEK1832 are indicated together with coordinates to IS3 on the side of the gels.

Determination of the Nucleotide Sequences of Overhangs at the 5'-Ends—Plasmid pSEK1832 is a type I miniplasmid derived from pSEK1831 used here carries IS3, which is flanked by the same sequences, 5'-AGG-3'/'3'-AGG-5', that are the target sequence duplicated upon IS3 insertion (Sekine et al., 1994). It is possible that the 3-nt overhangs are the target sequence.

To determine whether the linear molecules observed are of IS3 with 3-nt overhangs at the 5'-ends of IRR and IRL, which are supposed to be 5'-TCC-3' and 5'-AGC-3', respectively, we carried out sequencing analysis as follows. To determine the nucleotide sequence of the overhangs attached to the 5'-end of IRL, the linear IS3 molecules were first incubated with a modified T7 DNA polymerase (Sequenase), which lacks the 3' → 5' exonuclease activity, in the presence of [α-32P]dTTP. The DNA sample obtained was digested with BsmI, heat-denatured, and electrophoresed in a sequencing gel. As shown in Fig. 4A, a single-stranded DNA fragment, 106 nt in length, with an extension of 1 nt from the 3'-end of IRL was generated in the presence of [α-32P]dTTP but was not in the presence of either [α-32P]dATP, [α-32P]dCTP, or [α-32P]dGTP. This shows that only dGTP was incorporated and thus that the nucleotide next to the 5'-end of IRL in the 3-nt overhang is dC. Next, when the linear IS3 molecules were incubated in the presence of [α-32P]dGTP plus dATP or dTTP, the BsmI digestion of the sample did not generate any fragments larger than the fragment (106 nt) that was generated in the presence of [α-32P]dGTP alone (Fig. 4A). However, in this experiment, when dCTP was added instead of dATP or dTTP, BsmI digestion generated the 107-nt fragment, 1 nt larger than the 106-nt fragment (Fig. 4A). This shows that dCTP is incorporated after dGTP and thus that the nucleotide at the middle position in the 3-nt overhang is dG. Finally, when the linear IS3 molecules were incubated in the presence of dTTP in addition to [α-32P]dGTP and dCTP, BsmI digestion of the sample generated the 108-nt fragment, 1 nt larger than the product (107 nt).
that was generated in the presence of \([\alpha-32P]dGTP\) and dCTP (Fig. 4A). However, in this experiment, when dATP was added instead of dTTP, BsmI digestion did not generate such extension products (Fig. 4A). This shows that dTTP was incorporated after dGTP and dCTP and thus that the nucleotide at the 5'-end position in the 3-nt overhang is dA. All of these results show that the nucleotide sequence of the overhang attached to the 5'-end of IRL is 5'-AGC-3'. Note that this sequence is identical to the sequence flanking IRL of IS3 in the parental plasmid pSEK1832.

To determine the nucleotide sequence of the 3-nt overhang at the 5'-end of IRR, the linear IS3 molecules isolated were incubated with Sequenase in the presence of \([\alpha-32P]dNTP\) and digested with Ncol, which cleaves IS3 at one site. In the presence of \([\alpha-32P]dGTP\), Ncol digestion generated an extension product, 173 nt in length, 2 nt longer than that with the exact 3'-end of IRR (Fig. 4B). In the presence of \([\alpha-32P]dCTP\) or \([\alpha-32P]dTTP\), Ncol digestion did not generate such extension products, but in the presence of \([\alpha-32P]dATP\), Ncol digestion gave rise to two faint bands of the fragments, 172 and 173 nt in length (Fig. 4B). These results show that the nucleotide(s) next to the 5'-end of IRR in the linear IS3 molecules is dCC, dTT, or dT and that the molecules with the overhanging sequence dCC are major. When the linear IS3 fragments were incubated in the presence of \([\alpha-32P]dGTP\) plus dATP, an extension product, 174 nt in length, 1 bp longer than that generated in the presence of \([\alpha-32P]dGTP\) alone, and in addition, another extension product, 173 nt in length, forming a faint band, were generated (Fig. 4B). In this experiment, when dTTP or dCTP was added instead of dATP, the extension product of 173 nt long was generated, but the extension product of 174 nt long was not (Fig. 4B). However, when the three nucleotides, dCTP, dTTP, and dATP, were added in the presence of \([\alpha-32P]dGTP\), the extension product, 174 nt in length, was generated (Fig. 4B). These results show that the nucleotide sequence of the overhang at the 5'-end of IRR in the major linear IS3 molecules is 5'-TCC-3' and that in the minor ones is either 5'-VTT-3' (where V is A or G or C) or 5'-RCT-3' (where R is A or G), etc. Note that the overhanging sequence 5'-TCC-3' is identical to the sequence adjacent to IRR in the parental plasmid pSEK1832. We will discuss later the reason the linear fragments with overhanging sequences other than 5'-TCC-3' are generated.

**Discussion**

We have shown in this paper that the efficient production of IS3 transposase results in the generation of the linear IS3 molecules having 5'-ends with overhanging sequences of 3 nt. Nucleotide sequences of the 3-nt overhangs of the major linear IS3 molecules generated from pSEK1832 are identical to those flanking IS3 in the parental plasmid. This result indicates that the linear molecules are excised from the parental plasmid by staggered breaks at both ends regions of IS3 by the action of the transposase. In transposons Tn10 and Tn7, which transpose in a non-replicative manner, double strand breaks occur at both end regions of the elements to excise the linear transposon fragments, which are subsequently inserted into a target site (Morisato and Kleckner, 1984; Benjamin and Kleckner, 1989; Bainton et al., 1991). Identification and characterization of the linear IS3 molecules further support the previous notion that IS3 transposes in a non-replicative manner (Sekine et al., 1994).

As described under “Results,” some of the linear IS3 molecules were found to have the 3-nt overhanging sequences different from those flanking IS3 in the parental plasmid. We have observed here and previously (Sekine et al., 1994) that IS3 mediates deletion frequently in the region adjacent to IRR of IS3 to produce miniplasmids, which now have IS3 flanked by different sequences, and that these miniplasmids still generate many kinds of smaller miniplasmids. It is therefore quite likely that the linear IS3 molecules with different overhanging sequences were generated by excision from the smaller miniplasmids.

It should be noted that unlike Tn10 and Tn7, IS3 generates IS3 circles consisting of the entire IS3 sequence and a 3-bp sequence intervening between the IS3 ends (Sekine et al., 1994) (Fig. 5). Most of the IS3 circles contain the intervening 3-bp sequence, which is identical to either one of the sequences flanking IS3 in the parental plasmid, but others contain the 3-bp sequence different from the original sequences flanking IS3. The latter IS3 circles are supposed to be produced from miniplasmids with a flanking sequence different from that in the parental plasmid. It is likely that these IS3 circles are derived by circularization of the linear IS3 molecules (Fig. 5), such that only one 3'-end of IS3 is joined to the 5'-end of the 3-nt overhanging sequence on the other side, and the resulting 3-nt gap on the opposite strand is subsequently converted to a homoduplex form through DNA repair. Alternatively, both 3'-ends of IS3 are joined with the 5'-ends of the 3-nt overhanging sequence on the other side to give circles with a 3-bp heteroduplex sequence intervening between IRL and IRR. (If the 3-bp sequence flanking IS3 are identical, the circles should have a homoduplex sequence intervening between IRLs.) The IS3 circles with the sequence of one or the other strand could have been obtained by cloning. It is not clear at present whether the IS3 circles participate as substrates in transposition or not. Polard et al. (1992) have reported that an IS3 family element, IS911, also generates IS3 circles similar to the IS3 circles and that the IS911 circles are, however, not the obligatory transposition intermediates.

As described above, IS3 generates both circular and linear molecules, while Tn10 and Tn7 generate linear molecules but not circles. Retroviruses are, however, known to generate circular DNAs with two long terminal repeats in addition to double-stranded linear DNA molecules after reverse transcription from the viral RNA genome (Varum and Brown, 1989) (Fig. 5). In this respect, IS3 resembles retroviruses. The linear molecules of retroviruses are considered to be the intermediates for their integration, in which 2 nt from each 3'-end of the linear viral DNA are removed by integrase to produce 5'-protruding ends (Craigie et al., 1990; Katz et al., 1990; Katzman et al., 1989; Sherman and Fyfe, 1990), and the 3'-ends of the linear molecules are subsequently joined to the 5'-ends generated at a target site (Fujiwara and Mizuuchi, 1988; Brown et al., 1991). Identification and characterization of the linear IS3 molecules further support the previous notion that IS3 transposes in a non-replicative manner (Sekine et al., 1994).
al., 1989) (Fig. 5). Considering the conservation of the amino acid sequence motif in transposases of IS3 family elements and retroviral integrases, we assume that the linear IS3 molecules are the transposition intermediates and are inserted into a target site by a similar mechanism to that in the retroviral system. Probably, the 3'-OH of the linear IS3 molecule is joined to 5'-P of the target DNA, which is supposed to be generated by 3-bp staggered breaks (Fig. 5), since IS3 has been shown to give a 3-bp target duplication at its point of insertion (Sommer et al., 1979; Timmerman and Tu, 1985; Yoshioka et al., 1987; Spielmann-Ryser et al., 1991). The 3-nt gap on the opposite strand is subsequently repaired to convert the gap to a duplex form and to remove the 3-nt donor sequence attached to the 5'-end of the linear IS3 molecule (Fig. 5).

The linear IS3 molecules are accumulated to a level that can be readily detected in the DNA preparation from a small overnight culture by gel electrophoresis and staining the gel with ethidium bromide. This may imply that the ends of the linear IS3 molecules are protected from the attack of cellular nucleases. In Tn10, there exists such a protein-DNA complex, which is an active form of transposition intermediate (Haniford et al., 1979; Timmerman and Tu, 1985; Yoshioka et al., 1987; Spielmann-Ryser et al., 1991). It is likely that a gapped donor molecule, i.e., a donor backbone, is produced when IS3 has been excised. We have, in fact, detected such donor backbone molecules in the DNA sample in a smaller amount than that of the linear IS3 molecules. This suggests that the donor backbone molecules released do not form a protein-DNA complex and are thus subjected to the attack of nucleases unlike the linear IS3 molecules.

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