1. An *in vitro* System Involving Tn3 Transposase that Catalyzes the Formation of DNA Deletions

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The prokaryotic transposon Tn3 is known to cause deletion of DNA externally adjacent to its ends.^{1),2)} We have devised a system for quantitative measurement of frequency of such deletions and have shown that their occurrence depends on *tnpA*, a gene encoding the transposase, which is known to be essential for transposition, and thus concluded that such deletion may be regarded as intramolecular transposition.³⁾

Though two *in vitro* DNA rearrangement systems have thus far been constructed with other transposons^{4),5)} no such system that is dependent on the Tn3 transposase has been reported to date. We describe here an *in vitro* DNA deletion formation system by purified Tn3 transposase.

Materials and methods. Restriction endonucleases and DNA polymerase I were purchased from Takara Shuzo or from Boehringer Mannheim. Tn3 transposase was prepared as described previously,⁶⁾ except that phospho-cellulose chromatography was replaced by Pharmacia FPLC with Mono-S HR 5/5. Integration host factor (IHF) was purified from the overproducer strain K5746 as described by Nash *et al.*⁷⁾ For some of the later experiments (B–D in Table I), phospho-cellulose fraction was further purified by Phenyl-Sepharose (Pharmacia) chromatography.⁸⁾ Protein concentrations were determined with the Bio-Rad protein assay kit, with bovine serum albumin (Sigma, Fraction V) as standard.

Plasmid pMM200 was used as the substrate for deletion forming reaction (Fig. 1A). Cells harboring pMM200 are killed at 42°C, at which temperature the *cI857* repressor is inactivated and transcription from the *p_L* promoter is derepressed, inducing the *kil* gene activity. This process is valid unless the *kil* gene has been inactivated, either directly or indirectly, e.g. by deletion. We have shown that Tn3 transposase, when supplied in *trans* by an intact *tnpA* gene, can cause deletion in pMM200 *in vivo*.⁶⁾ Plasmid DNA was purified as described by Maniatis *et al.*⁹⁾

Standard *in vitro* reactions of deletion formation were carried out in a total volume of 20 μ l which contained 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 50 mM NaCl, 10% glycerin, 2 μ g pMM200 DNA, 1 to 1.2 μ g transposase, and 180 to 200 ng IHF. In the earlier stages of this study, DNA polymerase I, ATP and the 4 dNTP (dATP, dGTP, dCTP and dTTP) were included in the reactions. After incubation at 30°C for 60 min, reaction mixtures were vortexed with 180 μ l of 50 mM Tris-HCl buffer (pH 8.8) containing 0.22% sodium dodecyl sulfate, followed by shaking with 200 μ l of phenol saturated in 20 mM Tris-HCl (pH 8.0). After centrifugation (4000 rpm $\times$ 10 min), the water layer was passed through a Sephadex G100 column (0.2 cm² $\times$ 5 cm). The eluent fractions were used for transformation of C600 *recA*, which was kindly given by Dr. H. Ogawa. Transformation was carried out as described by Cohen *et al.*¹⁰⁾

Fig. 1. A: Structure of pMM200. The left end and the right end are connected to form a circle. pMM200 contains a modified Tn3 in which the two inverted repeats (IR) and the *bla* gene (gene for ampicillin resistance) are intact, but *tnpA* and *tnpR* have been inactivated by deletion. A λ DNA fragment including the *kil* and *cI857* genes is also contained in pMM200. Thick line and thin line represent DNA regions derived from pHK8³⁹ and λ *cI857*, respectively. The horizontal arrows above indicate the direction of transcription of each gene. Left(L) and right(R) inverted repeats of Tn3 are represented by triangles. Restriction sites are abbreviated as follows; E, *EcoRI*; R, *EcoRV*; B, *BamHI*; G, *BglII*; S, *SalI*; P, *PstI*; H, *HindIII*. The *EcoRV* site marked by the circle was retained in all the *in vitro* products examined (see Results section). B: Regions deleted in *in vitro* reaction products. Horizontal lines indicate the DNA regions whose absence could be shown by restriction analysis, and wavy lines indicate the regions within which the deletion end points must be located.

except for differences in temperatures. Transformants were selected on L-agar⁵⁾ containing 100 μ g/ml ampicillin (Ap). The deletion forming activity was estimated by the ratio of the number of Ap resistant transformants at 42°C to that at 30°C.

Results. The results of *in vitro* deletion forming reactions are shown in Table I. The activity was dependent on the Tn3 transposase, IHF and Mg^{++} . Since DNA polymerase I was known to be required for replication of ColE1-type plasmids,¹¹⁾ (and our substrate pMM200 belongs to this group), we initially included this enzyme, (and ATP and the 4 dNTP) in the reaction mixture. But removal of DNA polymerase I had no significant effect (B, C), or it even seemed to be inhibitory at times (D). Furthermore, the activity did not depend on ATP or 4 dNTP (D). The reaction products, after having been isolated from transformants, were examined by restriction analysis. Most of the plasmids examined (33 of 46) were shorter than pMM200 as shown by agarose gel electrophoresis and therefore must be deletion products. All the products examined (46) were found to retain the *EcoRV* site which is present between the *kil* gene and Tn3 left IR (see Fig. 1A); thus none of these deletions starts exactly from the end of Tn3. Thirteen deletion products were analyzed further by other restriction enzymes (Fig. 1B). Although all the plasmids examined had lost DNA regions including the *kil* gene or the *pL* promoter, starting points of the deletions were heterogeneous on both sides.

Table I. DNA deletion formation *in vitro*

A. Effect of IHF and transposase					
Transposase ($\mu\text{g/ml}$)	IHF ($\mu\text{g/ml}$)	Transformants		Ratio of transformants ($42^\circ\text{C}/30^\circ\text{C}$) $\times 10^5$	
		$30^\circ\text{C}\times 10^{-4}$	42°C		
0	0	4.3	2	4.7	
0	9	3.1	3	9.7	
2.5	0	4.8	3	6.3	
2.5	9	5.5	4	7.3	
10	0	6.3	0	<1.6	
10	9	3.8	1	2.6	
50	0	6.7	4	6.0	
50	9	7.8	18*	23.1	

B. Effect of IHF and DNA polymerase I						
Transposase ($\mu\text{g/ml}$)	IHF ($\mu\text{g/ml}$)	DNA poly- merase I (units)	Transformants		Ratio of transformants ($42^\circ\text{C}/30^\circ\text{C}$) $\times 10^5$	
			$30^\circ\text{C}\times 10^{-4}$	42°C		
B1, B2			B1, B2	B1, B2	B1,	B2
50, 60	10	0	16.8, 11.1	19, 16	11.3,	14.4
50, 60	0	0.5	13.2, 7.9	8, 5	6.1,	6.3
0, 0	10	0.5	17.3, 7.9	5, 2	2.9,	2.5
50, 60	10	0.5	12.5, 7.5	28*, 5	22.4,	6.7

C. Effect of IHF in the absence of DNA polymerase I						
Transposase ($\mu\text{g/ml}$)	IHF ($\mu\text{g/ml}$)	Transformants		Ratio of transformants ($42^\circ\text{C}/30^\circ\text{C}$) $\times 10^5$		
		$30^\circ\text{C}\times 10^{-4}$	42°C			
		C1, C2	C1, C2	C1, C2	Average	
60	0	5.5, 4.6	5, 3	9.1, 6.5	7.8	
0	10	6.4, 3.9	1, 1	1.6, 2.6	2.2	
60	10	6.3, 4.7	10, 8	15.9, 17.0	16.5	

D. Effect of ATP, 4 dNTP, DNA polymerase I and MgCl_2							
Trans- posase ($\mu\text{g/ml}$)	IHF ($\mu\text{g/ml}$)	Factors added or omitted	Transformants		Ratio of transformants ($42^\circ\text{C}/30^\circ\text{C}$) $\times 10^5$		
			$30^\circ\text{C}\times 10^{-4}$	42°C			
			D1, D2	D1, D2	D1,	D2	Average
0	0	none	13.4	4	3.0		
0	10	none	12.9	6	4.7		
60	0	none	13.0	9	6.9		
60	10	none	4.5, 14.5	11, 16	24.4,	11.0	17.7
60	10	+ATP	5.2, 13.6	5, 19	9.6,	14.0	11.8
60	10	+4 dNTP	4.5, 12.2	9, 11	20.0,	9.0	14.5
60	10	+4 dNTP, DNA- polymerase I	14.1	14	9.9		
60	10	+ATP, 4 dNTP	6.4	9	14.1		
60	10	+EDTA (25mM) -- MgCl_2	13.8	7	5.1		

Five hundred nanograms of DNA was used for each transformation assay. A small volume of transformed cell cultures was plated at 30°C . The rest of the cells was collected by centrifugation and similarly plated at 42°C . Values of the transformants at 42°C are the actual number of colonies. Transformants for equivalent volume were calculated for 30°C . Transformation with substrate DNA with no incubation gave an average of $(4.99\pm 0.79)\times 10^{-5}$ for the ratio of the transformants at 42°C to that at 30°C . DNA polymerase I (0.5 units) was included in all reactions in A and, when indicated, in B and D. ATP (2 mM) and the 4 dNTP, (75 μM each) were included in all reactions in A, B, and C and the indicated samples in D. Results of two separate reactions are shown in B, C, and D. Results shown in D2 are the sum of two separate transformation assays of a single reaction. Plasmids from all of the Ap and temperature resistant transformants indicated by * in A and B1 were purified and used for restriction pattern analysis shown in Fig. 1.

Discussion. The only biological activity reported thus far for purified Tn3 transposase is the DNA binding activity.^{(6),(11),(12)} Our finding in the present study that purified transposase mediates DNA deletion formation is the first report that describes an enzymatic activity of this protein. While transposase-dependent deletions, which may be regarded as intramolecular transposition,⁽³⁾ have been shown to occur invariably from one of the ends of Tn3 *in vivo*,^{(1),(2),(6)} in our *in vitro* reaction no product molecules with deletions starting exactly at an end of Tn3 have so far been observed. This discrepancy between the *in vivo* and *in vitro* systems cannot be explained at the present stage. Possibilities are that more protein component(s) and/or different reaction conditions are required for the specificity. However, that the *in vitro* reaction requires IHF, which is involved in another transposon-related DNA rearrangement⁽⁵⁾ or a transposase-DNA interaction,⁽¹³⁾ suggests that our *in vitro* system reflects at least some aspects of the *in vivo* process.

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