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In Vitro Transposition of Transposon Tn*3**

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Hitoshi Ichikawa‡ and Eiichi Ohtsubo

From the Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Transposition of the ampicillin-resistant transposon Tn*3* was reproduced *in vitro* using the *Escherichia coli* cell extract. In this cell-free system, we used plasmid DNA carrying mini-Tn*3* as donor and phage λ DNA as target and assayed for ampicillin-resistance transducing phages formed by cointegration of these DNA molecules. Ampicillin-resistance transducing phages, which were obtained by *in vitro* packaging of λ DNA after the *in vitro* transposition reaction, were formed only in the presence of Tn*3* transposase. The reaction required mini-Tn*3* with the proper sequence and orientation of the terminal inverted repeats of Tn*3*. The reaction also required DNA synthesis but not RNA synthesis by *E. coli* RNA polymerase.

Transposon Tn*3* (4957 base pairs (bp)[1] in length) transposes by a two-step mechanism (Heffron *et al.*, 1979; for reviews, see Grindley, 1988; Sherratt, 1989): The first step is formation of a cointegrate, in which a donor replicon carrying Tn*3* and a recipient replicon fuse together, and consequently Tn*3* is duplicated and appears at junctions between the two replicons in a direct orientation (Gill *et al.*, 1978; McCormick *et al.*, 1981). The cointegrate formation requires the *tnpA* gene product, transposase, which is encoded by Tn*3* itself (Gill *et al.*, 1979; Chou *et al.*, 1979; Heffron *et al.*, 1979; McCormick *et al.*, 1981) and the 38-bp inverted repeat (IR) sequences at both ends of Tn*3* (Huang *et al.*, 1986; Ichikawa *et al.*, 1990). The second step is to break down (resolve) the cointegrate to generate a recipient replicon having received a copy of Tn*3* as well as the original donor replicon with a copy of Tn*3*. This resolution step requires the *tnpR* gene product, resolvase, which is also encoded by Tn*3* (Kostriken *et al.*, 1981; Reed, 1981). This paper describes reproduction *in vitro* of cointegration, the primary step of Tn*3* transposition.

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‡ Recipient of a Fellowship of the Japan Society for the Promotion of Science for Japanese Junior Scientists. Present address: Saitama Cancer Center Research Institute, Ina-machi, Kitaadachi-gun, Saitama-ken 362, Japan.

[1] The abbreviations used are: bp, base pair(s); IR(s), terminal inverted repeat(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NTP, ribonucleotide 5′-triphosphate; dNTP, deoxyribonucleotide 5′-triphosphate; ddNTP, 2′,3′-dideoxyribonucleotide 5′-triphosphate; ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; ddATP, 2′,3′-dideoxyadenosine 5′-triphosphate; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; *amp*[r], ampicillin resistance/resistant; IRL, inverted repeat at the left terminus; IRR, inverted repeat at the right terminus.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Phage—The bacterial strains used were all *E. coli* K12 derivatives: D110*polA*$^+$ (*thyA endA*) was obtained from Dr. T. Itoh; JM109 (Yanisch-Perron *et al.*, 1985) is a laboratory stock. Ymel (λ^- *mel supF*) and its λ lysogen Ymel$(\lambda)^+$, and BHB2688 and BHB2690 were obtained from Dr. H. Ikeda, Tokyo University. The plasmids containing mini-Tn*3* (see Table I; Ichikawa *et al.*, 1987, 1990) were derivatives of plasmid pUC18 (Yanisch-Perron *et al.*, 1985). Plasmid pHS12*tnpA* and phage λ*b*519*N7nin*5 (87.2% of wild type λ in length) were described previously (Ichikawa *et al.*, 1990).

DNA Preparation—Plasmid DNA was isolated from JM109 harboring each plasmid and purified by ethidium bromide-CsCl density gradient centrifugation according to Ohtsubo *et al.* (1978).

Phage λ*b*519*N7nin*5 was grown in Ymel, and phage DNA was extracted with phenol from phage particles which were purified by CsCl density gradient centrifugation. To prepare concatemers of λ*b*519*N7nin*5 DNA which were used as target for mini-Tn*3* transposition, the DNA extracted was treated with T4 DNA ligase (Takara) at 37 °C for 2-3 h at a DNA concentration of 200 μg/ml, extracted gently with phenol, and dialyzed against TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA).

Preparation of Tn3 Transposase and E. coli Cell Extracts—Transposase was prepared as described previously (Ichikawa *et al.*, 1987).

The *E. coli* cell extract used for *in vitro* transposition was fraction II reported by Fuller *et al.* (1981) and was prepared, as follows: D110*polA*$^+$ harboring plasmid pHS12*tnpA* or D110*polA*$^+$ harboring no plasmid was grown at 30 or 37 °C, respectively, to OD_{595} = 1 in 2 liters of L broth (1% Bacto-tryptone (Difco), 0.5% Bacto-yeast extract (Difco), 0.5% NaCl, 2 mM NaOH) supplemented with 0.2% glucose and 20 μg/ml thymine, harvested, and resuspended in buffer A (25 mM NaHEPES, pH 7.6, 1 mM EDTA) to a final volume of 6 ml. The cell suspension was frozen in liquid nitrogen and stored at −70 °C. It was thawed at 2 °C, and 240 μl of 4 M KCl, 12 μl of 1 M dithiothreitol, and 60 μl of 25 mg/ml lysozyme were added. After incubation at 0 °C for 20 min, the suspension was frozen and thawed again. The cleared lysate was obtained by centrifugation at 200,000 × *g* for 20 min. Ammonium sulfate was added slowly at 0 °C to 48% saturation (280 mg/ml lysate), and the precipitate was collected by centrifugation at 48,000 × *g* for 20 min. The pellet was resuspended in 250 μl of buffer B (25 mM NaHEPES, pH 7.6, 0.1 mM EDTA, 2 mM dithiothreitol), and dialyzed against 80 ml of buffer B at 0 °C for 1 h. The dialysate (containing about 100 mg/ml protein) was frozen in liquid nitrogen and stored at −70 °C. Concentrations of proteins were determined using the method of Bradford (1976) with bovine serum albumin (Seikagaku Kogyo) as standard.

In Vitro Transposition Reaction—The standard reaction mixture (50 μl) contained 5 mg/ml protein of *E. coli* cell extract, 10 μg/ml plasmid DNA, 20 μg/ml λ*b*519*N7nin*5 DNA concatemerized, 25 mM NaHEPES, pH 7.6, 12 mM magnesium acetate, 5 mM dithiothreitol, 60 mM KCl, 2 mM ATP, 200 μM each of three NTPs (GTP, UTP, and CTP), 100 μM each of four dNTPs (dGTP, dATP, dTTP, and dCTP), 40 μM NAD, 50 μg/ml bovine serum albumin, 100 μg/ml tRNA, 2% polyvinyl alcohol (Sigma), 20 mM creatine phosphate, and 100 μg/ml creatine kinase (Sigma). Reaction was started by adding the *E. coli* cell extract and carried out at 30 °C for 90 min after preincubation of the reaction mixture at 0 °C for 10 min. The reaction was stopped by adding 200 μl of 10 mM EDTA.

To examine the requirement of ATP in the *in vitro* transposition reaction, ATP as well as creatine phosphate and creatine kinase were omitted from the standard reaction mixture. To examine the effect of ddNTPs (ddGTP, ddATP, ddTTP and ddCTP) (Amersham Corp.) in the reaction, dNTPs were omitted and replaced with 50 μM each of ddNTPs. To examine the effect of drugs, novobiocin sodium salt,

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oxolinic acid, or rifampicin (Sigma) was added to 100, 100, or 20 μg/ml, respectively. Oxolinic acid was added after neutralizing it with NaOH.

Assay for amp^r Transducing Phages—The reaction mixture was treated successively with phenol, phenol/chloroform, and chloroform, and the DNA in the aqueous phase was ethanol-precipitated, and resuspended in 5 μl of λ dilution buffer (10 mM Tris/HCl, pH 7.5, 10 mM $MgSO_4$). The DNA was packaged *in vitro* into phage particles using lysates prepared from BHB2688 and BHB2690 essentially according to Maniatis *et al.* (1982). Plaque-forming units of phages were measured using Ymel as indicator, and amp^r transducing phages were scored on plates containing 50 μg/ml ampicillin after infection of $Ymel(\lambda)^+$ with them, as described previously (Ichikawa *et al.*, 1990). Plasmids in the amp^r transductants were examined by electrophoresis in 0.7% agarose gels.

RESULTS

Cointegration Mediated by Mini-Tn3 in Vitro—We have previously constructed pUC18 derivatives by inserting the fragment of Tn*3* containing the left or right terminal inverted repeat (IRL or IRR, respectively) or synthetic IR of Tn*3* or of γδ, a transposon related to Tn*3*. These derivatives consequently contain mini-Tn*3*s which have the amp^r gene flanked by the inserted IR and the right Tn*3* terminal sequence (IRR) preexisting in pUC18 (Fig. 1 and Table I) (Ichikawa *et al.*, 1990). Mini-Tn*3* with wild-type IRs in the inverted orientation has been shown *in vivo* to mediate cointegration between the plasmid carrying mini-Tn*3* as donor and phage λ as target in the presence of transposase to form cointegrates, namely amp^r transducing phages with the duplication of the mini-Tn*3* sequences, yielding the amp^r transductants in a λ lysogen (Ichikawa *et al.*, 1990) which harbor large plasmids as well as the original donor plasmids produced upon recombination at the duplicated sequences. A mini-Tn*3* variant with wild-type IR inserted at an end in the direct orientation or with γδ IR in the inverted orientation abolishes or decreases the ability to mediate the cointegration (Ichikawa *et al.*, 1990; see the legend to Table I).

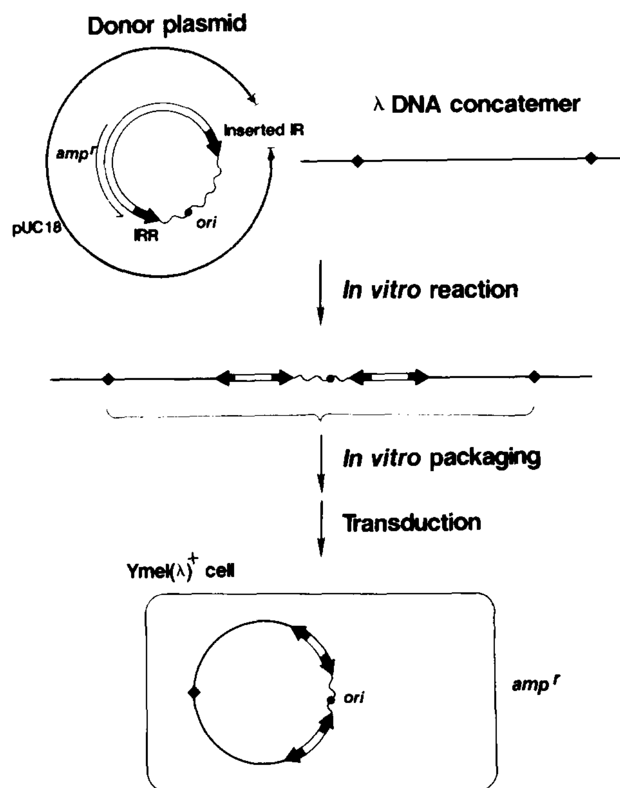

FIG. 1. **Structures of donor and target DNA molecules and the scheme of the λ packaging assay of *in vitro* transposition of Tn*3*.** The donor plasmid carries mini-Tn*3* containing IRR and an inserted IR (*thick solid arrows*) which flank the amp^r region (*thick open lines*). The non-mini-Tn*3* part in the plasmid is shown with a *wavy line*. After the cointegration reaction with λ DNA concatemer with *cos* sites (*solid boxes*) used as the target, DNA was purified and packaged *in vitro* into phage particles. These phage particles were mixed with a log phase culture of $Ymel(\lambda)^+$ and were plated after 1 h of incubation at 32 °C for scoring the amp^r transducing phages. Plaque-forming units in the *in vitro* packaged materials was assayed separately. We expect that the resulting amp^r transductants harbor large cointegrate plasmids with the duplication of the mini-Tn*3* sequence as well as the original donor plasmids which are generated upon recombination at the duplicated sequences in the cointegrates, as have been observed *in vivo*.

TABLE I

Donor DNA molecule requirement for in vitro transposition of mini-Tn3

Plasmid pUC18 originally contains the 1209-bp DNA segment of Tn*3* including IRR and the amp^r gene. pUC18L1 and pUC18L2 have an inserted 200-bp segment of Tn*3* including IRL in the inverted and direct orientations to IRR preexisting in pUC18, respectively. pUC18R1 contains an inserted 303-bp segment of Tn*3* including IRR in the inverted orientation to the preexisting IRR in pUC18. A pair of IRs in each plasmid except pUC18 flank the amp^r gene to create a mini-Tn*3* variant (see Fig. 1). *In vitro* transposition of the mini-Tn*3* was carried out as described under "Materials and Methods," using λ*b*519*N*7*nin*5 DNA as the target (see Fig. 1). The products (amp^r transducing phages) were assayed as shown schematically in Fig. 1. Note here that pUC18L1, pUC18R1, and pSIR24 form cointegrates with phage λ *in vivo* at frequencies 5.5~8.4 × 10^{-5} amp^r transducing phages per plaque-forming unit (pfu), while the other plasmids form the cointegrates at frequencies much less than 1 × 10^{-6} (Ichikawa *et al.*, 1990).

Plasmid	Inserted IR (orientation)	pfu	amp^r	amp^r/pfu (%)
		×10^7/ml	×10^3/ml	
pUC18	None	9.9	<0.05	<5.1 × 10^{-7} (<1.4)
pUC18L1	Tn3 IRL (inverted)	7.6	2.7	3.6 × 10^{-5} (100)
pUC18L2	Tn3 IRL (direct)	9.5	<0.05	<5.3 × 10^{-7} (<1.5)
pUC18R1	Tn3 IRR (inverted)	13.4	6.9	5.1 × 10^{-5} (140)
pSIR24	Tn3 IR (inverted)	11.6	6.0	5.2 × 10^{-6} (140)
pSIR26	γδ IRR (inverted)	9.9	0.50	5.1 × 10^{-6} (14)

To reproduce the cointegration reaction of Tn*3 in vitro*, we mixed the plasmid DNA and λ concatemer DNA with *cos* sites and incubated with the *E. coli* cell extract prepared from cells harboring plasmid pHS12*tnpA* which overproduces Tn*3* transposase under the conditions described under "Materials and Methods." DNA was purified and packaged into λ particles *in vitro*, and amp^r transducing phages were then assayed by selecting for amp^r transductants (see Fig. 1).

amp^r transductants were obtained when we used plasmid pUC18L1 which carries a mini-Tn*3* with IRL inserted into pUC18 in the inverted orientation to IRR preexisting in pUC18 (Table I). All 14 transductants examined contained large plasmids as well as the original donor plasmids, as observed *in vivo*, indicating that the reaction products are the amp^r transducing phages which are cointegrates with the duplication of the mini-Tn*3* sequences.

amp^r transductants were also obtained when we used plasmid pUC18R1 or pSIR24, which carries a mini-Tn*3* with IRR or synthetic IR, respectively, inserted into pUC18 in the inverted orientation to IRR preexisting in pUC18 (Table I), but no amp^r transductants were obtained when we used either pUC18L2 carrying a mini-Tn*3* variant with IRL inserted in pUC18 in the direct orientation to IRR preexisting in pUC18, or pUC18, carrying only one IR (Table I). pSIR26, which carries a mini-Tn*3* variant with IR of γδ having seven base substitutions as compared with Tn*3* IR, showed a reduced frequency in production of the amp^r transductants (Table I).

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These results are consistent with those obtained previously *in vivo* (Ichikawa *et al.*, 1990).

In this cell-free system, Tn*3* transposition reaction had a long time lag of about 60 min and needed about a 120-min incubation to reach a maximum efficiency (Fig. 2).

Requirements of the Reaction—When the extract prepared from cells harboring no pHS12*tnpA* was used, no *amp*^{r} transducing phages were formed (Fig. 3). However, addition of the purified transposase in the reaction mixture gave rise to *amp*^{r} transducing phages, and the appearance of the transducing phages was proportional to the amount of transposase added (Fig. 3). This shows that formation of the transducing phages is dependent on transposase.

The reaction required Mg^{2+}, ATP, and an ATP regenerating system (Table II). A hydrophilic polymer, polyvinyl alcohol, was also required, but the high concentration of polyvinyl alcohol (more than 4%) inhibited the reaction (data not shown). Another hydrophilic polymer, polyethylene glycol, was also effective for the reaction but inhibited it under the high concentration of polyethylene glycol, like polyvinyl alcohol (data not shown).

Omission of dNTPs had a small effect, but addition of ddNTPs in place of the dNTPs inhibited the reaction (Table II). This indicates that DNA synthesis is required for the reaction. Addition of the DNA gyrase inhibitors novobiocin and oxolinic acid also inhibited the reaction (Table II), indicating that DNA gyrase is involved in the reaction. Omission of ribonucleotide triphosphates (GTP, CTP, and UTP) as well as addition of the RNA polymerase inhibitor rifampicin, however, did not inhibit the reaction (Table II). This indicates that RNA synthesis by RNA polymerase is not required.

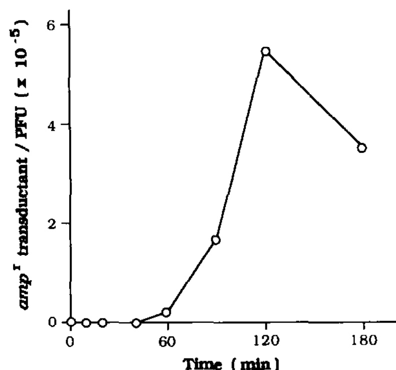

FIG. 2. **Time course of *in vitro* reaction.** The reaction contained pUC18L1 DNA as donor and λ concatemer DNA as target. Conditions were as described under "Materials and Methods." Extract from cells harboring pHS12*tnpA* was contained in the reaction mixture. *PFU*, plaque-forming units.

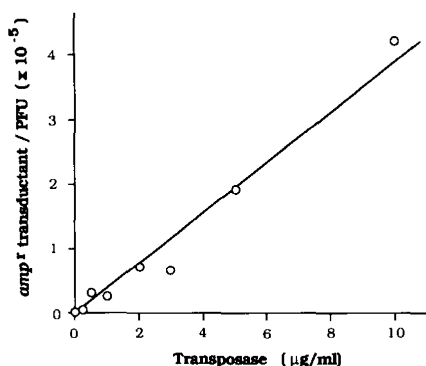

FIG. 3. **Dependence on Tn*3* transposase in *in vitro* transposition.** The reaction contained pUC18L1 DNA as donor and λ concatemer DNA as target. Conditions were as described under "Materials and Methods." Extract from cells harboring no pHS12*tnpA* and various amount of transposase were contained in the reaction mixture. *PFU*, plaque-forming units.

TABLE II

Requirements of in vitro transposition and effects of inhibitors in the reaction

Reaction conditions were as described under "Materials and Methods." pfu, plaque-forming units.

Condition	pfu	*amp*^{r}	*amp*^{r}/pfu (%)
	$\times 10^7$/*ml*	$\times 10^2$/*ml*	
Complete	2.6	6.0	2.3×10^{-5} (100)
$-Mg^{2+}$	24.8	<0.3	$<1.2 \times 10^{-7}$ (<0.5)
−ATP	27.3	<0.3	$<1.1 \times 10^{-7}$ (<0.5)
−GTP, CTP, UTP	3.0	9.5	3.2×10^{-5} (140)
−dNTPs	2.0	1.5	7.5×10^{-6} (33)
Complete	2.7	6.3	2.3×10^{-5} (100)
−dNTPs	2.7	3.0	1.1×10^{-5} (48)
−dNTPs, +ddNTPs	1.4	<0.3	$<2.1 \times 10^{-6}$ (<9.1)
+Novobiocin	1.3	<0.3	$<2.3 \times 10^{-6}$ (<10)
+Oxolinic acid	4.3	<0.3	$<7.0 \times 10^{-7}$ (<3.0)
+Rifampicin	1.9	6.5	3.4×10^{-5} (150)

DISCUSSION

In this paper, we have described a cell-free system capable of generating *amp*^{r} transducing phages formed between the plasmid carrying mini-Tn*3* and phage λ. This sytem required mini-Tn*3* with a proper structure in respect to IRs and depended on transposase, demonstrating that mini-Tn*3*-mediated cointegration, the primary step of transposition, has occurred *in vitro*.

Our cell-free system included fraction II which allows replication of the OriC plasmid, containing replication origin of the *E. coli* chromosome (Fuller *et al.*, 1981). We used this, because the cointegration event must be accompanied with replication of the Tn*3* DNA. The reaction was in fact inhibited by inhibitors of DNA synthesis, ddNTP, novobiocin, and oxolinic acid. Hydrophilic polymer such as polyvinyl alcohol was also required for the reaction, as observed in replication of the OriC plasmid (Fuller *et al.*, 1981). This may suggest that DNA synthesis required for the *in vitro* transposition reaction occurs using some of the *E. coli* proteins which are used for OriC plasmid replication. RNA synthesis by RNA polymerase was, however, not required. This indicates that the DNA synthesis required in our cell-free system is not initiated by a primer RNA(s) generated by RNA polymerase, unlike that in the replication system of pUC18 (or mini-Tn*3* donor plasmid), whose replication is dependent on RNA synthesis by RNA polymerase.

The reaction showed a long time lag of about 60 min. It is likely that this time lag may be due to the multi-step mechanism involved in transposition of Tn*3*, in which the transposition complex is assumed to be formed at IRs with transposase and probably with a host protein(s) in the donor molecules and then interacts with the target molecules prior to the strand transfer reaction (Lee *et al.*, 1983a, 1983b; Ichikawa *et al.*, 1990; Amemura *et al.*, 1990).

Transposition of phage Mu (Mizuuchi, 1983) and transposon Tn*10* (Morisato and Kleckner, 1987) has been reproduced *in vitro*. Mu performs two types of transposition, cointegration and simple insertion; the former involves replication of the entire segment of Mu (replicative transposition), but the latter does not (conservative transposition) (Mizuuchi, 1984). Tn*10* transposes in a manner of simple insertion (Bender and Kleckner, 1986). Tn*3* is different from Mu or Tn*10*, since transposition of Tn*3* is almost exclusively replicative. We are

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currently trying to obtain transposition intermediate molecules made *in vitro* and to analyze their structures to understand the molecular mechanism of Tn*3* transposition by cointegration.

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