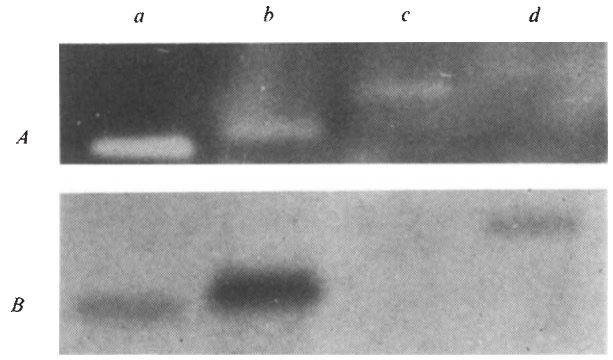

Fig. 4 Strand separation of *Kpn*I 1.2 and 1.5 kb DNA segments and hybridization with ^{32}P-labelled nuclear RNA synthesized *in vivo*. ***A*** Shows a stained gel in which the two complementary strands of the inserts of two of the *Kpn*I plasmids, purified as described below, have been run in separate lanes. Lanes *a* and *b* contain the fast and slow strands, respectively, of the pBK(1.2)11 insert, and lanes *c* and *d* contain the fast and slow strands of the pBK(1.5)54 insert. ***B*** Shows the corresponding autoradiograms, obtained after hybridization to *in vivo* labelled nuclear RNA.
Methods: 11 μg of the plasmids pBK(1.2)11 and pBK(1.5)54 were restricted with *Kpn*I, ethanol precipitated and resuspended in 100 μl of a solution containing 30% (v/v) dimethyl sulphoxide, 1 mM EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue. The samples were heated at 97 °C for 3 min, chilled in ice-water, and electrophoresed at 6 V cm^{-1} for 4 h in a 1% agarose gel in 50 mM Tris-HCl, *p*H 8.4, 0.38 M glycine, and 2 mM EDTA[27]. Agarose strips containing the separated strands were frozen in an ethanol dry-ice bath and then thawed at 55 °C for 10 min. This procedure was repeated five times. The agarose slurry was centrifuged at 3,000*g* for 10 min and the supernatant removed and saved. The pellets were extracted twice with 0.5 M NH_4-acetate and the combined supernatants then adjusted to 0.5 M with NH_4-acetate. DNA was then ethanol precipitated in the presence of 10 mM $MgCl_2$ and 10 μg ml^{-1} of purified yeast tRNA. The pellets were vacuum dried, resuspended in water containing 15% sucrose, 0.4% orange G, 2.4 mM EDTA and 0.02% SDS. The complementary strands were electrophoresed in separate lanes in a 1% agarose–TBE buffer gel system (Fig. 2). The DNA was transferred to dibenzyloxymethyl (DBM) paper in 1 M Na-acetate as described by Christophe *et al.*[28]. After transfer, the DBM filter was washed once in 0.5 N NaOH and twice in 2 × SSC, each for 30 min at room temperature. The filters were hybridized with 10^7 c.p.m. of ^{32}P-labelled nuclear RNA labelled *in vivo* as described in the legend to Fig. 2. Hybridization conditions, washes and exposure times were as described in Fig. 3.

*Kpn*I family members were immobilized on nitrocellulose filters and hybridized with ^{32}P-labelled RNA synthesized *in vitro* in the presence or absence of α-amanitin. A human *Alu* sequence cloned in the *Bam*HI site of pBR322 (Blur 8) and a human β-actin cDNA sequence served as controls. Figure 3*A* shows that in the absence of α-amanitin, transcripts of the *Alu* sequences (lane *g*), the β-actin gene sequences (lane *h*) and all but one (that represented by plasmid pBK(1.8)20, lane *d*) of the *Kpn*I families (lanes *a–f*) were synthesized.

Figure 3*B* shows that 0.6 μg ml^{-1} α-amanitin inhibited transcription of all but one of the *Kpn*I sequences, and of the β-actin gene. *Alu* sequences and sequences homologous to pBK(1.9)51, however, continued to be transcribed, although at a reduced rate. Plasmid pBK(1.9)51 actually contains an *Alu*-like sequence[8], so it was not possible to determine which of the RNA polymerases was responsible for the transcription of the *Kpn*I sequences in this plasmid. 200 μg ml^{-1} of α-amanitin inhibited transcription of all the *Kpn*I sequences and the *Alu* sequences. Thus, unlike the *Alu* family sequences, which are transcribed extensively by RNA polymerase III[16], the *Kpn*I family sequences are transcribed by RNA polymerase II. These results suggest that either polymerase II promoters exist in *Kpn*I DNAs or that their transcription occurs by extension of RNA polymerase II transcripts initiating upstream from the sequences. In the latter case, *Kpn*I repeats must be close to these polymerase II transcription units since nascent chains extend only about 500 bases in the *in vitro* system[17].

That the polymerase transcribes the *Kpn*I sequences asymmetrically was indicated by the following experiment. The separated, complementary strands of the inserts in pBK(1.2)11 and in pBK(1.5)54 were hybridized to HeLa nuclear RNA labelled *in vivo* with ^{32}P-orthophosphate. As shown in Fig. 4, the labelled RNA hybridized predominantly with the slow strands of both inserts. Experiments with RNA labelled with ^{32}P-UTP in the *in vitro* system also indicated asymmetric transcription of both strands. In this case, RNA sequences homologous to the slow strands of *Kpn*I 1.2 and 1.5 kb DNAs were 2.5–3.5 times more abundant than transcripts homologous to the corresponding fast strands.

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DNA sequences at the ends of transposon Tn*5* required for transposition

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Transposons are a class of genetic elements that can move from one site in a cell's genome to another independently of the cell's general recombination system. Little is known about the mechanism of transposition of compound transposons such as Tn*5*, but it is thought that a transposon-encoded protein (a transposase) must recognize the outer ends of the element and, together with host factors, catalyse the transfer of the internal DNA into a new site in a manner that may involve replication. It has previously been shown that the synthesis of an IS*50R*-encoded protein (protein 1) is an essential requirement for Tn*5* transposition[1–5]. Here we demonstrate that a structure containing only the outer 186 base pairs (bp) of both inverted repeats is capable of being efficiently complemented to transpose in *Escherichia coli*, provided IS*50R* is located close by on the same replicon. In addition, *Bal*31-generated deletions indicate that 16–18 bp of the outer end of IS*50L* are required for

transposition. This 16–18-bp sequence contains the 8-9-bp small inverted repeat present at each end of IS*50* plus a 9–bp sequence which is homologous to an interrelated sequence present in four copies in the chromosomal origin of replication in a variety of Gram-negative bacteria. This sequence organization suggests that the ends of Tn*5* may function to provide a recognition site for the Tn*5* transposase adjacent to a sequence recognized by the host replication system.

Tn*5* is an example of a composite prokaryotic transposable element. It contains two physically similar but genetically different 1,534-bp insertion sequences (IS*50R* and IS*50L)* in inverted orientation flanking a 2.7-kilobase (kb) segment of unique DNA which contains a gene encoding an aminoglycoside antibiotic modifying function[6–9]. A derivative of Tn*5* was constructed containing the outer 186 bp of the element in inverted orientation flanking a 2-kb segment of DNA encoding tetracycline resistance. While this structure is unable to transpose in the absence of a functional copy of IS*50R*, transposition of Tn*5-320* from a ColE1 plasmid to λ was detected when a wild-type copy of Tn*5* was present on an F plasmid[3]. This complementation was very inefficient, as Tn*5-320* only transposed at 1–3% the frequency of the wild-type element. Table 1 shows that complementation remains inefficient when the donor Tn*5* is present on a multi-copy plasmid. However, when the wild-type donor is located on the same replicon within 420 bp of Tn*5-320*, both elements transpose at approximately equal frequencies. This result indicates that Tn*5-320* contains all the DNA sequence information required for efficient translocation when the transposase is synthesized in close proximity. The inability of the transposase to act efficiently in *trans* has been demonstrated in several studies with Tn*5* (refs 2–4) and other Tn*5*-like transposable elements (for example, Tn*10*, Tn*903*, IS*1*)[10–13] and may be due to an unstable nature of the active form of the transposase or a high affinity for DNA at the site of synthesis followed by a limited one-dimensional diffusion along the DNA[13].

To investigate the sequence requirements for transposition further, we varied the amount of sequence present in the left inverted repeat (IS*50L*) while leaving the functional right repeat (IS*50R)* intact. Table 2 shows that efficient transposition occurs in derivatives such as Tn*5–341* and Tn*5-346* which contain only the outer 56 bp of the left inverted repeat. In addition, the sequence immediately adjacent to the left repeat, including the 9 bp of duplicated host DNA[8], which is different in Tn*5-341* and Tn*5-346*, has little effect on subsequent transposition.

pRZ341 was constructed so that a unique *Bam*HI restriction site was located at nucleotide 53 from the left end of the element. By linearizing pRZ341 at the *Bam*HI site, treating with *Bal*31 exonuclease, and re-ligating in the presence of *Bam*HI107ntaining oligonucleotides (see Table 3), we were able to remove progressively the remaining sequences in the left inverted repeat. Subsequent transposition assays revealed that deletions up to nucleotide 21 (Tn*5-341:21* actually contains the outer 23 bp of Tn*5* as the first 2 bp of the *Bam*HI linker is equivalent to nucleotides 22 and 23 of Tn*5*) had no effect on the transposition frequency (Table 3). An element (Tn*5-341: 18*) containing only the outer 18 bp is still able to transpose although at a reduced frequency. However, removal of an additional 3 or more bp (Tn*5-341:15, 13* or *7*) results in a drastic lowering of the transposition proficiency of the element to the point where it is undetectable in our assays.

Table 1 Complementation of Tn*5-320*

Exp	Tn*5* elements present	Transposition frequency Tet^r	Kan^r	Tet^r/Kan^r
A	ColE1::Tn*5-320*	$<6.6\times10^{-9}$	—	—
B	ColE1::Tn*5-320* + RSF1010::Tn*5*-wt	4.6×10^{-7}	1.1×10^{-5}	0.04
C	ColE1::Tn*5-320:* Tn*5*-wt	4.8×10^{-6}	5.2×10^{-6}	0.92

Cultures of RZ502λbbn (*E. coli* W3110 *lac recA56 srl str* λ*cI*857 *b515 b*519 *nin*5 *S*am 7)[3] containing the various plasmids were heat induced and the resulting lysates used to lysogenize Hf1–1 as described previously[1]. The transposition frequency measures the kanamycin (Kan = Tn*5*-wild-type, wt) or tetracycline (Ter = Tn*5-320*) resistant transductants per plaque-forming unit of the phage. Expt *A*, no IS*50R* functions are supplied; expt *B*, IS*50R* functions are supplied on the multi-copy compatible plasmid RSF1010; expt *C*, wild-type Tn*5* was transposed onto ColE1::Tn*5-320* and is located ~420 bp from Tn*5-320* with IS*50R* oriented adjacent to Tn*5-320*. In expt *C*, 3% of the Kan^r transductants were also Tet^r and 11% of the Tet^r transductants were also Kan^r due to transposition of Tn*5-320*::Tn*5*-wild-type. The doubly resistant transductants were not included in the values shown. Tn*5-320* was constructed as elsewhere described[3]. Wild-type Tn*5* was transposed onto RSF1010 by Jerry Yin.

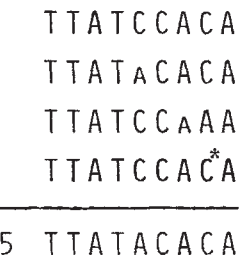


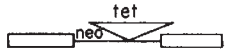
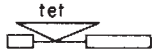
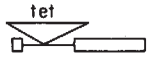
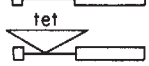
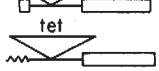
Fig. 1 Homology at the ends of Tn*5* with a repeated sequence in the chromosomal origin of replication (*oriC*). The Tn*5* sequence from nucleotides 8–16 is given with the four copies (R1–R4) of the interrelated sequence in the *oriC* region of six different Gram-negative bacteria[14,15] A large letter indicates that the nucleotide is totally conserved; a small letter indicates it is the predominant nucleotide. C* indicates the location of a single base pair change in the *E. coli oriC* which inactivates its function[20].

These results indicate that the outer 16–18 bp of DNA are essential for efficient transposition, although maximum transposition may require sequences up to nucleotide 23. The outer 9 bp of IS*50* are repeated with one mismatch in inverted orientation at the inner end of the insertion sequence[8,9], which has led to the proposal that this sequence may constitute at least part of the recognition determinant for the IS*50R*-encoded transposase[13,21]. It is perhaps surprising that Tn*5-341:15* and Tn*5-341:13*, which contain the terminal 9 bp sequence, are unable to transpose since both IS*50* or a dimeric Tn*5* containing a direct repeat structure transpose at relatively high efficiency[9,21]. This indicates that an inner end of IS*50* can substitute for one outer end to mediate transposition even though it contains only the terminal 8/9 bp in common with the outer end. We have not yet investigated the sequence requirements of an IS*50* inner end nor do we know whether the transposition process of IS*50*-like elements is mechanistically identical to a normal Tn*5* structure.

The sequences adjacent to the terminal 9 bp repeat (nucleotides 7–16) display remarkable similarity to a highly homologous 9-bp sequence which is repeated four times within the *oriC* region of six different Gram-negative bacteria (Fig. 1)[14,15]. The significance of this homology is suggested by the difference in the transposition proficiency of Tn*5-341:18* which leaves this sequence (plus an additional 2 bp) intact, contrasting with the transposition-defective Tn*5-341:15* in which the last base pair of this sequence is absent. The *oriC* homologous sequence is not present at the inner end of IS*50*. However, Isberg *et al.*[2] and Berg and co-workers[9,21] have concluded that the outer and inner ends of IS*50* are not functionally symmetrical. Isberg *et al.*[2] determined that a pair of inner ends mediated transposition at $<0.2\%$ the frequency of the outer ends. In contrast, similar studies have shown that the inner and outer ends of the structurally related transposon Tn*10* do function with equal efficiency[10].

The *oriC*-like sequence found at the outer ends of Tn*5* may serve to direct host replication functions to the ends of the element during transposition. The requirement for this sequence at the end distal to the site of transposase synthesis suggests that transposition of Tn*5* may involve replication

Table 2 Transposition assays of Tn*5* derivatives containing various amounts of the left inverted repeat

Plasmid present	Structure of Tn*5*	Left end sequence present	Transposition frequency
pRZ190		1–1,534	9.4×10^{-6}
pRZ191		1–118	1.1×10^{-5}
pRZ341		1–56	1.8×10^{-5}
pRZ346		1–56	1.8×10^{-5}
pRZ340		0	$<5.0\times10^{-9}$

Transposition assays of Tn*5* derivatives containing various amounts of the left inverted repeat. Transposition assays were performed in RZ102λ bbn (RZ102 = *E. coli* MO *recA56 str*)[3] as described in Table 1 legend. pRZ190 is ColE1-Tn*5* containing a 2.7-kb *Bgl*II restriction fragment which carries the tetracycline resistance determinant inserted into the *Bam*HI site in Tn*5* (S. J. Rothstein and W.S.R., unpublished). pRZ191 has the DNA from the *Sal*I site in pRZ190 to the *Hae*III site in the left inverted repeat deleted[5]. pRZ341 was constructed by ligating a 120-bp *Eco*RI-*Sau*3a restriction fragment, which contains the outer 56 bp of IS*50L* plus ColE1 sequences, from pRZ102[7] into pBR322 between the *Eco*RI site and the *Bam*HI site, regenerating the *Bam*HI restriction site. An *Eco*RI-*Sal*I fragment from this plasmid was then substituted for the left inverted repeat of pRZ190. pRZ346 was made in an analogous manner starting with a Tn*5* insertion into pBR322 with IS*50R* located in the tetracycline resistance region about 45 bp from the *Eco*RI site. Therefore, the left end of Tn*5-346* is actually derived from IS*50R* and is adjacent to pBR322 sequences. pRZ340 contains non-Tn*5* DNA at its left end and was constructed by ligating an *Eco*RI-*Sal*I fragment from pRZ4006 (a pBR322 derived plasmid containing the *lacPO* between the *Eco*RI and *Bam*HI sites) into pRZ190.

Table 3 Transposition assays on *Bal*31 generated deletions into the left inverted repeat of Tn*5*

	Tn*5* mutant	Transposition frequency
1 → 10 20 30 CTGACTCTTATACACAAGTAGCGTCCTGAA	Tn*5-341*	1.7×10^{-5}
.....CGGGA	Tn*5-341:25*	1.3×10^{-5}
.....CGGGATCCC	Tn*5-341:21*	1.7×10^{-5}
.....CGGGATCCCG	Tn*5-341:18*	2.8×10^{-6}
.....CGGGATCCCG	Tn*5-341:15*	$<1.0\times10^{-9}$
.....CGGGATCCCG	Tn*5-341:13*	$<1.0\times10^{-9}$
.....CGGGATCCCG	Tn*5-341:7*	$<1.0\times10^{-9}$

Transposition assays on *Bal*31-generated deletions into the left inverted repeat of Tn*5*. The nucleotide sequence of the outer 30 bp of Tn*5* is given along with the location of the *Bam*HI containing oligonucleotide (in italics) present in the deletion mutants. A dot indicates the wild-type nucleotide is present. The arrow depicts those bases which are repeated in inverted orientation at the inner end of IS*50* and the underlined sequence contains the nucleotides which are homologous to a conserved sequence found in four copies in the *oriC* region (see Fig. 1). The transposition assays were performed in RZ201λ bbn as described in the Table 1 legend. The deletion mutants were constructed as follows. pRZ341 (Table 2) was linearized at its *Bam*HI restriction site and subjected to *Bal*31 exonuclease (New England Biolabs) digestion at 1 U ml^{-1} in a 100-μl reaction volume containing 0.2 M NaCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 1 mM EDTA, 20 mM Tris-HCl, *pH* 7.9, and 2.4 pmol of DNA termini. The reaction was terminated after 30–60 s at 30 °C by the addition of diethyl pyrocarbonate and EDTA to 0.05% and 30 mM, respectively. After ethanol precipitation, ~0.2 pmol of termini were ligated overnight at 15 °C in a 30–50 μl reaction volume with 24 pmol of *Bam*HI containing oligonucleotides (New England Biolabs) which were phosphorylated using polynucleotide kinase and ATP. The reaction mixture was then heated to 65 °C for 10 min to inactivate any remaining ligase and digested with 30 U of *Bam*HI for 4 h. The reaction was again heated to 65 °C for 10 min and the plasmid DNA containing *Bam*HI sticky ends was circularized in the presence of T4 DNA ligase (gift of R. Simoni) at a plasmid terminus concentration of approximately 1 nM and transformed into RZ211 (Δ(lac-pro) *recA56 srl str)*. The exact end points of the deletions were determined using the DNA sequencing methods of Maxam and Gilbert[19].

proceeding from both ends in a symmetrical manner. This type of mechanism may differ from the mechanism of transposition of other elements which is believed to involve polarized replication beginning at the end proximal to the site of transposase synthesis (for example, Tn*9* (ref. 16)). Perhaps this difference is reflected in the very low frequency of co-integration events promoted by Tn*5* in the absence of homologous recombination, in contrast to many other transposons (including Tn*9*)[4,17,18]. We have searched the outer 100 bp of several other transposable elements, including Tn*10*, *IS1*/Tn*9*, Tn*3* and bacteriophage Mu, and have found no significant homology to the *oriC* repeated sequence.

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