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Tn*2501*, a Component of the Lactose Transposon Tn*951*, Is an Example of a New Category of Class II Transposable Elements

THOMAS MICHIELS,[1] GUY CORNELIS,[1]* KIM ELLIS,[2] AND JOHN GRINSTED[2]

Unité de Microbiologie, Université Catholique de Louvain, UCL 54.90, B 1200 Brussels, Belgium,[1] and Department of Microbiology and Unit of Molecular Genetics, University of Bristol, Bristol, United Kingdom[2]

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Tn*2501* is a cryptic class II transposon found as part of the lactose transposon Tn*951*. Insertional inactivation and nucleotide sequence analysis of Tn*2501* allowed us (i) to localize the transposase (*tnpA*) and the resolvase (*tnpR*) genes as well as the resolution site (*res*) of Tn*2501* and (ii) to compare Tn*2501* with other well-known elements of the two subgroups of class II transposons (Tn*3*, γδ, Tn*951*, IS*101*; and Tn*21*, Tn*501*, Tn*1721*). The genetic organization of Tn*2501* is similar to that of Tn*3* with divergent transcription of the *tnpA* and *tnpR* genes away from the intervening *res* site. The *tnpR* gene of Tn*2501* shows weak homology with that of Tn*3* and even less with those of Tn*21* and Tn*501*. However, the *tnpA* gene and the inverted repeat sequences of Tn*2501* present more homology with those of Tn*21* and Tn*501* than with those of Tn*3*. Complementation studies showed that TnpA$^-$ mutants of Tn*2501* can be complemented, at a low frequency, by the Tn*21* transposase. None of the tested transposons complemented TnpR$^-$ mutants of Tn*2501*.

Tn*2501* is a 6.3-kilobase cryptic transposable element that was originally found as part of the lactose transposon, Tn*951* (4, 18). Bacterial transposable elements have been divided in two classes, depending on their structure and mode of transposition; Tn*2501* clearly falls into class II, the Tn*3* family (for a review, see reference 10). Thus, Tn*2501* has short inverted repeat sequences (IRs) that are related to those of Tn*3*, and it normally transposes via cointegrate intermediates (18). However, the sequence of the IRs of Tn*2501* indicates that it does not fall into either of the two well-defined groups of class II elements (the Tn*3* group and the Tn*21* group) but may well be a member of a previously unknown group (18).

In this paper, we report on a more detailed characterization of the transposition apparatus of Tn*2501*. In particular, the genes responsible for the process were located and partly sequenced. The data show that, as suspected, Tn*2501* is a member of an entirely new group of class II elements which has characteristics of both the Tn*3* group and the Tn*21* group.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* strains and the plasmids used are listed in Table 1. Strains containing plasmids were constructed by conjugation and transformation as appropriate, using standard methods. Plasmids were maintained in *E. coli* KL131.

Construction of Tn*2501* mutants. Plasmid pTM2 is a recombinant of Tn*2501* with a Tc^s derivative of pBR322 obtained by deletion of the 346-base-pair (bp) *Hin*dIII-*Bam*HI fragment (Fig. 1). Derivatives of pTM2 were constructed by inserting the *neo* gene of Tn*5* into various sites of the plasmid. The source of the *neo* gene was pGV707, which is pBR322 with the *Hin*dIII fragment of Tn*5* inserted in the *Hin*dIII site; this plasmid can be used as source of the *neo* gene on a *Hin*dIII, *Bam*HI, or *Sal*I fragment. In general, the appropriate *neo* fragment was inserted in pTM2 that had been partially digested with *Hin*dIII, *Bam*HI, or *Sal*I. For pTM8 and pTM9, the *Bam*HI *neo* fragment was inserted into a *Sma*I and an *Sst*II site, respectively; here *Bam*HI and *Sst*II ends were made blunt ended with the Klenow enzyme (see below) before ligation.

Construction of recombinants of pACYC184 with Tn*2501*, Tn*501*, Tn*21*, Tn*1721*, and Tn*3*. Recombinants were obtained by mobilization of pACYC184 by recombinants of R388 or F′ *lac* with the appropriate transposable element. All the resulting plasmids (PTM10 to pTM14) were, in fact, dimers of pACYC184 carrying one copy of the element.

Construction of pTM15. The *tnpA* gene of Tn*3* was cloned as the 4.8-kilobase *Eco*RI fragment from pFH18 (12) into the *Eco*RI site of pACYC184.

Growth and selection of bacteria. In general, bacteria were grown in tryptic soy broth medium, solidified with agar when necessary. For selection of bacteria, minimal salts agar was used. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 10 to 20 μg/ml; kanamycin, 50 μg/ml; tetracycline, 10 μg/ml; trimethoprim, 50 μg/ml. In all matings, donor strains were counterselected by using the relevant auxotrophic markers.

DNA preparation and manipulation. DNA was prepared either by CsCl-ethidium bromide gradient centrifugation or by a rapid method adapted from those of Grosveld et al. (9) and of Ish-Horowicz and Burcke (13). Digestions with restriction enzymes were done according to the recommendations of the manufacturer. Ends of fragments generated by restriction enzymes were filled in, when necessary, with the Klenow fragment of the DNA polymerase I, and ligations were performed by standard methods (15). Southern hybridization was performed as described by Cornelis and Saedler (5).

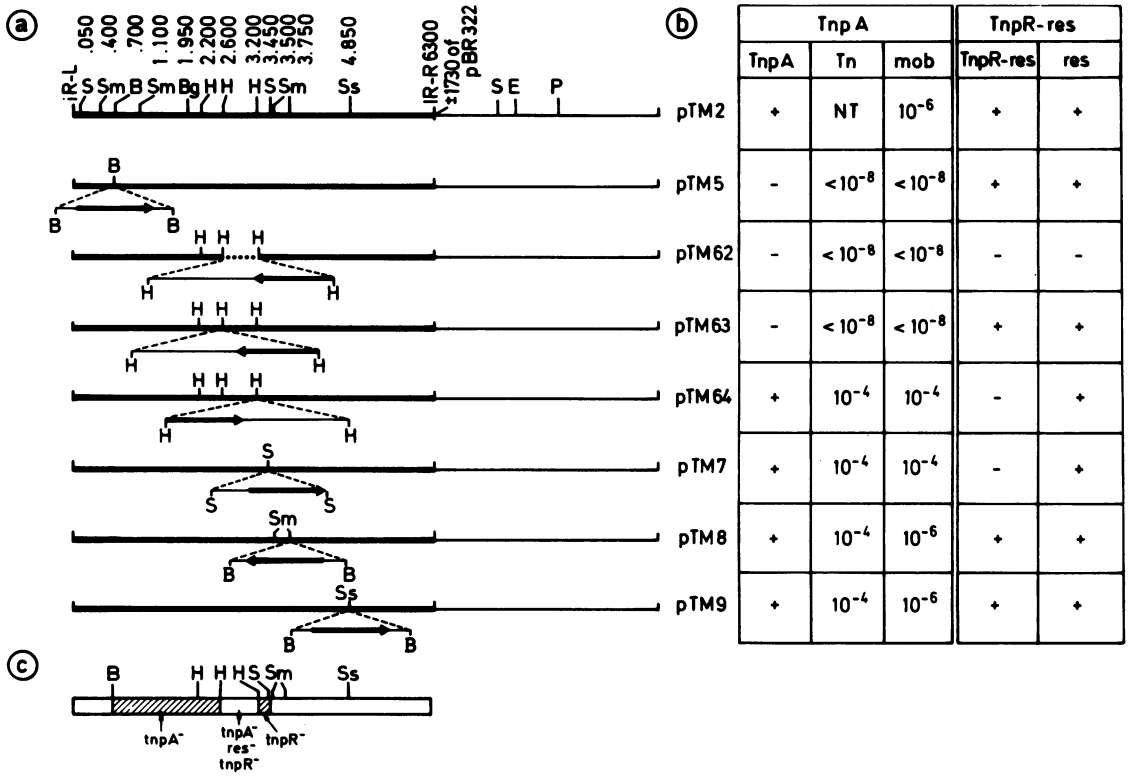
DNA sequencing. DNA was sequenced by both the dideoxy chain termination method (20) and the chemical method (16). In the former case, relevant restriction fragments were cloned in the M13 vectors mp10 and mp11 (17)

* Corresponding author.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant markers	Origin (reference)
KL131 (= CGSC 4332)	*leu arg met aro recA1*	14
JC6310	*his lys trp ΔlacX74 recA56 rpsL*	23
pTM2	Ap	pBR322 Δ*Hin*dIII-*Bam*HI::Tn*2501*
pTM5	Ap Km	pTM2-*neo* TnpA$^-$
pTM62	Ap Km	pTM2-*neo* TnpA$^-$ TnpR$^-$ Res$^-$
pTM63	Ap Km	pTM2-*neo* TnpA$^-$
pTM64	Ap Km	pTM2-*neo* TnpR$^-$
pTM7	Ap Km	pTM2-*neo* TnpR$^-$
pTM8	Ap Km	pTM2-*neo* TnpA$^+$ TnpR$^+$ Res$^+$
pTM9	Ap Km	pTM2-*neo* TnpA$^+$ TnpR$^+$ Res$^+$
pTM10	Tc Cm	pACYC184::Tn*2501*[a]
pTM11	Tc Cm Hg	pACYC184::Tn*501*[a]
pTM12	Tc Cm	pACYC184::Tn*1721*[a]
pTM13	Tc Cm Hg Sm Su	pACYC184::Tn*21*[a]
pTM14	Tc Cm Ap	pACYC184::Tn*3*[a]
pTM15	Tc	pACYC184-*tnpA* of Tn*3*
R388	Tp Su Tra$^+$	6
pGV707	Ap Km	pBR322-*neo* of Tn*5* (gift of J. Leemans)
pACYC184	Tc Cm	3

[a] pTM10 to pTM14 are dimers of pACYC184 containing one copy of the transposon.



	TnpA			TnpR-res	
	TnpA	Tn	mob	TnpR-res	res
pTM2	+	NT	10^{-6}	+	+
pTM5	-	$<10^{-8}$	$<10^{-8}$	+	+
pTM62	-	$<10^{-8}$	$<10^{-8}$	-	-
pTM63	-	$<10^{-8}$	$<10^{-8}$	+	+
pTM64	+	10^{-4}	10^{-4}	-	+
pTM7	+	10^{-4}	10^{-4}	-	+
pTM8	+	10^{-4}	10^{-6}	+	+
pTM9	+	10^{-4}	10^{-6}	+	+

FIG. 1. Genetic analysis of Tn*2501*. (a) Restriction map and localization of the mutations. Plasmid pTM2 is a derivative obtained by the insertion of Tn*2501* into a pBR322 derivative with the 346-bp *Hin*dIII-*Bam*HI fragment deleted. Insertion occurred around coordinate 1730 of pBR322. Mutants were constructed by insertion of a fragment containing the *neo* gene as described in Materials and Methods. Arrows represent the *neo* gene; dots represent deletions. Restriction sites are *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Sal*I (S), *Sma*I (Sm), and *Sst*II (Ss). (b) Phenotype of the mutants. TnpA indicates the TnpA phenotype, Tn indicates the transposition frequency, mob shows the transposition-associated mobilization frequency, TnpR-res indicates the presence or absence of resolution, and res indicates the presence or absence of the resolution site. The transposition and mobilization frequencies are calculated as described in Materials and Methods. (c) Genetic map of Tn*2501*. *tnpA* is the transposase gene, *tnpR* is the resolvase gene, and *res* is the resolution site.

before sequencing. In the latter case, fragments were cloned into plasmid pUC9 (22); they were then excised, using sites in the polylinker, and 3′ end labeled with ^{32}P-labeled nucleotides by repair of sites with the Klenow enzyme.

Assay of transposition functions. Class II transposable elements encode two enzymes that are involved in transposition: these are transposase (encoded by *tnpA*) and resolvase (encoded by *tnpR*). Transposase is involved in recognition of the IRs at the ends of the element, leading to the formation of cointegrates of donor and recipient transposons separated by directly oriented copies of the element. These cointegrates are then resolved into the original donor replicon plus the new recombinant by the action of the resolvase at the *res* sites in the elements.

Transposase activity was tested by assaying transposition of element-encoded markers to the transferable plasmid R388. Since the wild-type Tn*2501* has no marker, its transposase activity was compared with that of the mutants by checking their ability to promote the mobilization of the pBR322 derivative by R388. Thus, derivatives of KL131 containing R388 and the donor plasmid to be tested were constructed and then mated with *E. coli* JC6310 on the surface of a tryptic soy agar plate. Growth was scraped off the plates, and suitable dilutions were then plated on appropriate selective plates. These contained trimethoprim (to measure total transfer frequency of R388), kanamycin (to measure the frequency of transposants transferred), or ampicillin (to measure the frequency of cointegrates transferred; ampicillin is the marker on the donor replicon).

For the complementations of the transposase, the donor strains contained, in addition to R388 and the pTM2 derivative, a pACYC184 derivative carrying the complementing element. All experiments were done at least three times, and at least three transconjugants from each mating were analyzed with restriction enzymes to check that their structure was as predicted. Resolvase activity was checked by examining the stability of cointegrates isolated in the experiments described above. Thus, Amp^r transconjugants from the experiments above were mated with strain KL131, and Tp^r transconjugants were selected. Forty-eight of these were then picked on ampicillin: Amp^s indicated that resolution had occurred. Lack of resolution can be due to either TnpR$^-$ or Res$^-$ mutations. To distinguish these, wild-type Tn*2501* was introduced in *trans* to act as a source of resolvase; if the original lesions were TnpR$^-$, these would be complemented by the wild-type gene so that the cointegrates could now resolve, but if they were Res$^-$, resolvase would have no effects on cointegrates. Experimentally, the Amp^r transconjugants that proved to contain stable cointegrates were transformed with pTM10 (pACYC184::Tn*2501*), and then the resolution test performed again. Attempts at complementation of *tnpR* mutants with other elements were carried out in exactly the same way: Amp^r transconjugants that had been shown to be TnpR$^-$ were transformed with pACYC184 recombinants carrying the transposon to test, and then the stability of the cointegrates was rechecked.

Direct transposition. A low proportion of transposition events involving TnpR$^-$ and Res$^-$ mutants of Tn*3* and Tn*21* do not proceed via cointegrate intermediates. This phenomenon has been called direct transposition (1). Two TnpR$^-$ mutants of Tn*2501* were checked for direct transposition. Strain KL131 containing R388 and either pTM64 or pTM7 was mated with *E. coli* JC6310 as in the transposition assay (see above). Transconjugants selected on kanamycin were picked on ampicillin. Amp^s transconjugants arise by direct transposition.

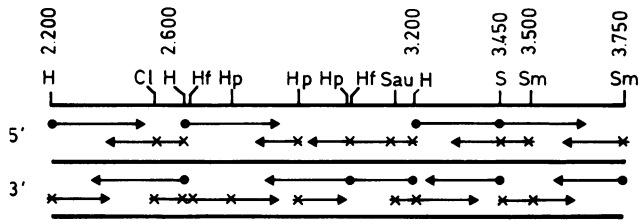


FIG. 2. Sequencing strategy. Relevant restriction fragments were sequenced either by the chain termination method (●→) or by the Maxam and Gilbert method (16) after 3′ end labeling with the Klenow enzyme (*→). Restriction sites are *Cla*I (Cl), *Hin*dIII (H), *Hin*fI (Hf), *Hpa*II (Hp), *Sal*I (S), *Sau*3a (Sau), and *Sma*I (Sm). Only the relevant *Hin*fI, *Hpa*II, and *Sau*3a sites are shown. Coordinates are as in Fig. 1.

RESULTS

Localization of transposition functions. Derivatives of a plasmid that contains Tn*2501* were constructed by insertion of a fragment of DNA that contains the *neo* gene of Tn*5* into various of the restriction enzyme sites (see Materials and Methods). The mutants were tested for *tnpA*, *tnpR*, and *res* functions (Fig. 1). These data define the extent of the *tnpA* gene as being from between the *Hin*dIII sites at coordinates 2.6 and 3.2 to after the *Bam*HI site at coordinate 0.7. Thus, insertion at the *Bam*HI site (pTM5) or at the *Hin*dIII site at 2.6 (pTM63) or replacing the *Hin*dIII fragment between 2.6 and 3.2 (pTM62) all resulted in complete lack of transposition. The transposition of these mutants was fully restored when complemented in *trans* by a wild-type Tn*2501* present on pTM10. An insertion at *Hin*dIII site 3.2 (pTM64) and all other insertions after that site retained transposition proficiency.

The extent of the *tnp*R gene was located as being from between the *Hin*dIII sites at 2.6 and 3.2 to between the *Sal*I site at 3.45 and the *Sma*I site at 3.75. Thus, the insertions at the *Hin*dIII site at 2.6 (pTM63) and the *Sma*I site at 3.75 (pTM8) were TnpR$^+$, while the insertions at the *Hin*dIII site at 3.2 (pTM64) and at the *Sal*I site at 3.45 (pTM7) were TnpR$^-$, as was the insertion that replaced the *Hin*dIII fragment between 2.6 and 3.2 (pTM62). This latter mutant was also the only one giving cointegrates that were stable in the presence of resolvase in *trans*. So the *res* site is probably located in this fragment. This 600-bp *Hin*dIII fragment thus contains one end of *tnpA*, one end of *tnpR*, and *res*. This implies strongly that the arrangement of the genes is the same as in Tn*3*, with *tnpA* and *tnpR* divergently transcribed with *res* between them.

The transposition frequency of the TnpA$^+$ TnpR$^+$ mutants (pTM8 and pTM9), determined by plasmid mobilization, was the same as that of the wild-type Tn*2501* (pTM2), excluding any effect of the presence of the *neo* gene on transposition.

TnpA$^+$ TnpR$^-$ mutants form cointegrates at a frequency that appears to be of the same order as the transposition frequency of TnpR$^+$ derivatives. The resolvase has thus no clear transposition repressor activity.

Direct transposition. Two TnpA$^+$ TnpR$^-$ Res$^+$ mutants (pTM64 and pTM7) were checked for direct transposition as described in Materials and Methods. Both transpose in 3 to 5% of the cases without producing cointegrate structures.

DNA sequence analysis. The region extending from the *Hin*dIII site at coordinate 2.2 to the *Sma*I site at 3.75 was sequenced (coordinates are as in Fig. 1). The sequencing strategy is shown in Fig. 2, and the sequence deduced is

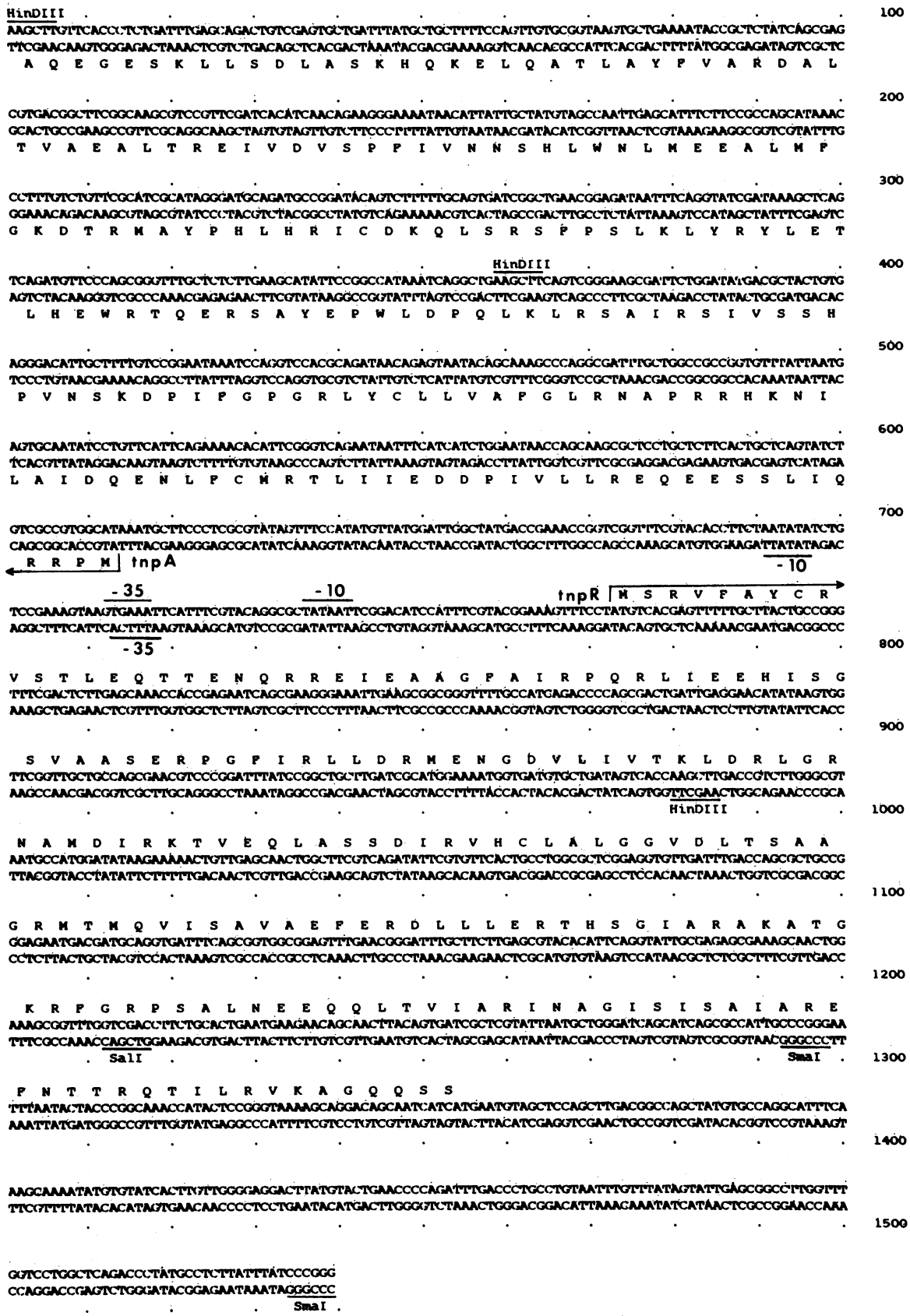


FIG. 3. Sequence of *tnpR*, *res*, and 600 bp of *tnpA* of Tn*2501*. Amino acid sequences of the transposase and of the resolvase, deduced from the DNA sequence, are shown in one-letter code. Arrows indicate the starting point of the *tnpA* and *tnpR* genes. Putative promoters are underlined. Relevant restriction sites are shown. The first *Hin*dIII site corresponds to the *Hin*dIII site at coordinate 2.2 on the map of pTM2 (Fig. 1). The *Sma*I site at the end of the sequence is the *Sma*I site at coordinate 3.75 in Fig. 2.

FIG. 4. Comparison of the transposase and resolvase of Tn*3*, Tn*21*/*501*, and Tn*2501*. Amino acid sequences are derived from Fig. 3 for Tn*2501*, from Heffron et al. (11) for Tn*3*, from Brown et al. (2) for Tn*501*, and from Diver et al. (7) for Tn*21*. *, Identical residues at a particular site. (A) N terminus of transposase (204 amino acids). For Tn*3*, single amino acids have been looped out at three positions. (B) Resolvase. Paddings of sequences to maximize homology are indicated by dashes.

shown in Fig. 3. Examination of the sequence shows that there are two divergent open reading frames in precisely the positions predicted by the genetic analysis. Thus, there is an open reading frame that starts within the *Hin*dIII fragment at 2.6 to 3.2 in the sequence shown in Fig. 1 and extends backwards reaching the end of the sequenced region without a stop, as predicted for the *tnpA* gene. And there is an open reading frame that starts within the same *Hin*dIII fragment and extends to between the *Sal*I site and the *Sma*I site at 3.75, as predicted for the *tnpR* gene.

The assignment of nucleotides 613 to 0 in the sequence shown in Fig. 3 to the start of the *tnpA* gene is strongly supported by the similarity of the resulting amino acid sequence with the start of the *tnpA* genes of Tn*3* and Tn*501* (2, 11) (Fig. 4A; see Discussion). There is no obvious strong promoter sequence immediately preceding this region; however, the region 718 to 683 is a possibility, with a −35 ATTTCA (compared with a consensus of TTGACA) and a −10 TATATT (compared with a consensus of TATAAT). There is also a G+A-rich region just before the initiation codon that could be a ribosome recognition sequence.

The putative *tnpR* gene starting at coordinate 773 in Fig. 3 and ending at 1354 could code for a protein of 194 amino acids with a molecular weight of 21,393. The assignment of this sequence to the *tnpR* gene is supported by the similarities between the resulting amino acid sequence and the sequences of the resolvase of Tn*3* and Tn*21* (7, 11) (Fig. 4B; see Discussion). A good promoter for this gene could be the

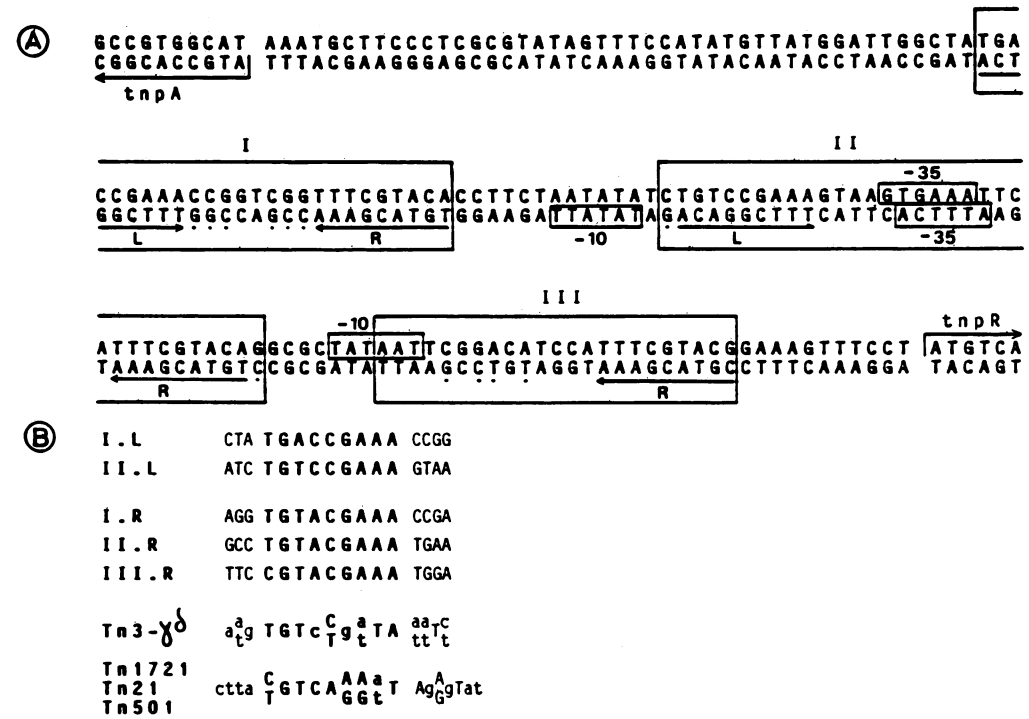


FIG. 5. Organization of the putative *res* site of Tn*2501*. (A) Sequence of the intergenic region. Dyad symmetry regions are framed. Nine base pairs are repeated in both orientations (L and R). Dots underline nucleotides that extend dyad symmetry. −10 and −35 boxes of the putative *tnpA* and *tnpR* promoters are boxed. (B) Comparison of the putative resolvase binding site of Tn*2501* with the corresponding part of the consensus sequences deduced from Tn*3* and γδ (8) and from the well-known class II transposons (19). Bold-face letters correspond to the 9-bp repeats, upper-case letters indicate well-conserved sequences, while lower-case letters indicate less-conserved sequences.

region between 712 and 748: the suggested −35 sequence (Fig. 3) is four-sixths homologous with the consensus and the −10 sequence is a perfect TATAAT, with a possible starting nucleotide of the transcript being an A in CAT. A possible ribosome recognition site of CGGAAA is present 8 bp before the initiation codon.

The genetic analysis indicated that *res* is completely contained within the *Hin*dIII fragment defined by coordinates 360 to 981 in the sequence shown in Fig. 3. Examination of the intergenic region in this sequence revealed a 9-bp repeated sequence creating three regions of imperfect dyad symmetry, similar to those seen in the *res* sites of Tn*3*, Tn*501*, and Tn*21* (8, 19). These regions are shown in Fig. 5A, and the comparison with other elements is shown in Fig. 5B (see also Discussion).

Complementation of transposition functions of Tn*2501* by other elements. The sequence deduced above indicates that Tn*2501* is less than 50% homologous with Tn*3* or Tn*21*. This lack of homology extends over the whole of the region that encodes transposition functions, as indicated by the lack of hybridization of the *Sal*I fragment from 0.05 to 3.45 (Fig. 1) to pTM11 (Tn*501*), pTM12 (Tn*1721*), pTM13 (*Tn21*), and pTM14 (Tn*3*) (data not shown). (These experiments would have detected homology of more than 60%.) Even so, however, the fact that Tn*2501* is obviously a class II element led us to check whether other class II elements could complement Tn*2501* transposition functions.

Plasmid pTM5 contains the *neo* fragment inserted into the *Bam*HI site of Tn*2501*; this transposon is TnpA$^-$ TnpR$^+$ Res$^+$ (see above). Complementation of the *tnpA* lesion was attempted with various class II elements (See Materials and Methods). Tn*2501* itself efficiently complemented transposition of the TnpA$^-$ mutant (frequency about 10^{-4}). Among the other elements tested (Tn*3*, Tn*21*, Tn*501*, Tn*1721*), only Tn*21* complemented the transposition of the TnpA$^-$ mutant. The frequency of this effect was between 10^{-8} and 10^{-7}. These data would appear to be consistent with the observation that the IRs of Tn*2501* were most closely related to those of Tn*21* (18).

Complementation of the resolvase function of pTM7 and pTM64 (both TnpA$^+$ TnpR$^-$ Res$^+$; see above) was attempted with various elements as described in Materials and Methods. Tn*2501* itself complemented resolution very efficiently. However, Tn*3*, Tn*21*, Tn*501*, and Tn*1721* were totally ineffective.

DISCUSSION

Class II transposable elements contain two well-defined groups, the Tn*3* group and the Tn*21* group (10). These groups have been defined on the basis of complementation studies, of the sequence of their IRs, of the comparisons of the sequences of the genes that encode transposition functions, and of the relative direction of transcription of the *tnpA* and *tnpR* genes. Tn*2501* is clearly a class II element, as shown by the sequence of its IRs and the mechanism of its transposition (18). But the sequence of its IRs does not place it clearly in either of the known groups of class II elements (although the IRs are more closely related to those of Tn*21* than to those of Tn*3* (18). The data presented here show that Tn*2501* has to be considered as a member of a previously unknown group of class II elements.

The homology between the transposition functions of Tn*2501* and those of Tn*3* and Tn*21* is very low; it is less than 50% overall in the sequenced region, and according to Southern hybridization, less than 60% in the rest. One end of the *tnpA* gene of Tn*2501* lies at coordinates 2.85 (Fig. 1), and assuming that there is just one gene, the other end lies between 0 and 0.7 (Fig. 1). The DNA sequence that was determined shows an open reading frame that starts 254 bp upstream from the *Hin*dIII site at 2.6 and extends beyond the *Hin*dIII site at coordinate 2.2 (Fig. 3). That this is indeed the beginning of the *tnpA* gene is confirmed by the similarity of the deduced amino acid sequence with that of the start of the *tnpA* gene of Tn*3* and Tn*501* (Tn*501* is closely related to Tn*21*) (Fig. 4A). With elements in the Tn*3* and the Tn*21* groups, the end of the *tnpA* gene actually lies within the IR; in the case of Tn*2501*, there is a termination codon in precisely the same relative position in the left IR (18), and it seems likely that this is the end of the *tnpA* gene in Tn*2501*. The comparison with the *tnpA* gene of Tn*3* shows 24% identity of amino acid residues, after taking into account the fact that the two sequences are not exactly aligned (the Tn*3* sequence has three insertions compared with the Tn*2501* sequence). The comparison with Tn*501* shows that the first 204 amino acids align exactly, and there is a 40% homology. Comparison with the sequence of Tn*21* itself (E. Ward and J. Grinsted, unpublished data) also shows exact alignment and even better homology (42% of the amino acids are identical). The close relationship of the *tnpA* gene of Tn*2501* with that of Tn*21* mirrors the situation with the IRs and fits in nicely with the fact that only Tn*21* complemented a *tnpA* mutant of Tn*2501*. It is clear, then, that as far as transposition itself goes (i.e., transposase plus IRs), Tn*2501* is most closely related to Tn*21* of the well-analyzed elements, although this relationship is not paticularly close.

The Tn*2501* resolvase is considerably different from those of Tn*3* and Tn*21*; considerable padding is required to align the sequences, and even then the overall homology is less than 35%. These differences are reflected in the fact that *tnpR* mutants of Tn*2501* were not complemented by Tn*3* or by Tn*21*. It seems that there might be slightly more homology with Tn*3*; this is more noticeable in the N-terminal region, where the homology is up to 35% with Tn*3* and 28% with Tn*21*.

The genetic analysis showed that the *res* site of Tn*2501* is in the *Hin*dIII fragment between coordinates 2.6 and 3.2 (Fig. 1). The *tnpA* and *tnpR* genes also start in this segment; taking Tn*3* as the example, it would thus be expected that *res* lies between these two genes. Figure 5A shows this intergenic region. In elements from both the Tn*3* group and from the Tn*21* group, the *res* site consists of three regions of imperfect dyad symmetry (8, 19). Three such regions can be seen in Tn*2501* (Fig. 5). A consensus for the repeated sequences is also shown in Fig. 5B; it can be seen that it is not closely related to those of either Tn*3* or Tn*21*, as might be expected in view of the large differences in the resolvase proteins that, of course, recognize the *res* sites.

There can be no doubt that Tn*2501* is the prototype of another group of class II transposable elements. But it is not clear how it fits into the pedigree of class II elements proposed by Schmitt et al. (21). This suggests that the Tn*3* and the Tn*21* groups diverged long ago when the *tnpR* gene was inverted and that the whole transposition apparatus of each group has evolved as a single unit. However, Tn*2501* has features of both Tn*3* (divergent transcription of *tnpA* and *tnpR*, and *tnpR* possibly more closely related to Tn*3*) and Tn*21* (the sequence of *tnpA* and of the IR). This almost suggests that *tnpA* and *tnpR* evolved separately and were joined together subsequent to the original divergence of the element from the others. How this could occur is not known, and in view of the precise correspondence in the relative position of *tnpA* and *tnpR* between Tn*2501* and the other elements, it does seem rather unlikely (unless there is a specific site [*res*?] at which *tnpR* genes can be inserted or exchanged).

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