*Gene*, 96 (1990) 43–49
Elsevier



GENE 03791

# The *virD* genes from the *vir* region of the Ti plasmid: T-region border dependent processing steps in different *rec* mutants of *Escherichia coli*

(Recombinant DNA; binary system; endonuclease; recombination; single-stranded DNA)

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Received by T.A. Bickle: 14 June 1990
Accepted: 10 August 1990

SUMMARY

We evaluated the substrate requirements for *virD*-mediated T-circle formation in an in vivo binary test system in *Escherichia coli*. Two copies of the 25-bp sequence which defines the right border of the T-DNA (transferred DNA) are sufficient, and the right and the left copy of the border are equivalent in function in this system. Experiments with different *rec* mutants show that the occurrence and frequency of circular double-stranded and single-stranded T-DNA equivalents strongly depend on *rec* functions of the host. These results are discussed in the context of processing of the tumor-inducing Ti plasmid preceding the T-DNA transfer from agrobacteria to plants.

## INTRODUCTION

*Agrobacterium tumefaciens*, known as the soil bacterium responsible for crown-gall disease, has one unique feature: it is able to transform plant cells by transfer of a part of its Ti plasmid (the T-DNA) with help of a second Ti plasmid region (*vir*-region) (for reviews see Zambryski, 1988; Ream, 1989). In the last few years, several laboratories have focussed on characterizing functions involved in inter-kingdom gene transfer. However, the exact steps by which the DNA is processed and integrated into the plant genome have yet to be characterized.

The initial processing steps of the Ti plasmid into a T-DNA transport form require two functions: (*i*) the 25-bp imperfect direct repeats which define the borders of the T-DNA, and (*ii*) the expression of the *vir*-region functions VirD1 and VirD2. However, depending on the experimental approach, different T-DNA processing forms have been obtained with different frequencies: (1) molecules specifically nicked either at one or at both borders (Yanofsky et al., 1986; Albright et al., 1987; Wang et al., 1987); (2) ss DNA corresponding to the bottom strand of T-DNA (Stachel et al., 1986; 1987; Albright et al., 1987; Jayaswal et al., 1987) with *virD2*-encoded protein covalently bound at the 5′ end (Young and Nester, 1988; Ward and Barnes, 1988; Dürrenberger et al., 1989); (3) ds linear T-DNA molecules (Jayaswal et al., 1987; Veluthambi et al., 1987; Steck et al., 1989), and (4) circular ds T-regions (Koukolikova-Nicola et al., 1985; Alt-Mörbe et al., 1986; Machida et al., 1986; Yamamoto et al., 1987; Timmermann

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Abbreviations: bp, base pair(s); Cm, chloramphenicol; DMSO, dimethylsulfoxide; ds, double strand(ed); kb, kilobase(s) or 1000 bp; LB, left border; nt, nucleotide(s); ORF, open reading frame; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; RB, right border of the T-region (for standard map of Ti plasmids); ss, single strand(ed); Tc, tetracycline; T-circle, circularized T-DNA or T-DNA equivalent, defined by the border sequences; T-DNA, transferred DNA; $T_L$, left portion of the split octopine type T-DNA; Ti, tumor inducing; VirD1 and VirD2, proteins expressed from the first and second ORF within the *virD* locus; *virD*, ORF encoding VirD proteins; wt, wild type.

et al., 1988). All of these forms were found in *vir*-region induced *Agrobacterium* as well as in *E. coli* cells expressing *virD*, and all were found by several authors with different approaches. This argues that they are not likely to be artifacts, but rather might represent intermediates which are produced successively or alternatively for different purposes from the Ti plasmid molecule.

Accordingly, different models for T-DNA transfer are discussed: (*1*) nicking of bottom strands at the borders followed by strand displacement and synthesis of new T-strand molecules result in ss T-DNA intermediates that might be transferred by a conjugative mechanism (Stachel et al., 1986; Albright et al., 1987), or (*2*) ds linear (Steck et al., 1989) or circularized ds T-regions which serve as the transport form. The first free T-DNA molecules detected *in planta* (Bakkeren et al., 1989) were shown to be linear (ss or ds). This does not exclude, however, a biological significance for circular forms detected in the bacteria.

We investigated initial T-region processing steps with a previously described binary test system in *E. coli* (Alt-Mörbe et al., 1986). It consists of one plasmid carrying VirD1/VirD2 functions and a second plasmid harboring their target sequence (the T-DNA border(s)). Here we study two aspects of VirD1/VirD2 action: (*i*) the influence of structural features of the substrate plasmid on T-circle production, and (*ii*) the interaction with recombination pathways during the formation of circular ds and of ss DNA products.

## RESULTS AND DISCUSSION

### (a) Nopaline Ti plasmid *virD*-triggered T-circle formation from different substrate plasmids

We have shown before (Alt-Mörbe et al., 1986) that expression of *virD1/virD2* causes circularization of a region within a plasmid which is defined as T-region by a right and left copy of the border sequences. To elucidate the mechanism of T-circle formation, we constructed a series of new substrate plasmids from the elements shown in Fig. 1. The different constructions and the corresponding results with and without the VirD1/VirD2-encoding plasmid pVIR78.2 are summarized in Fig. 2.

Very low mutation rates, no effect of the Vir function, and no uniform processing products were observed with substrate plasmids pS112.17 and pS130.11 which contain one synthetic RB as the only Ti-plasmid-derived sequence. In contrast, *virD* enhanced elimination of the *galK* cassette from pS130.8, which also carries only one border, but in addition a 322-bp direct repeat of vector sequences on both sides of the T-DNA equivalent. Addition of an LB on the left side of the T-DNA equivalent (pS131.4) still enhanced *virD* action. The products from plasmids pS130.8 and pS131.4 were indistinguishable. The junction occurred within one repeat, and the RB was retained. These results indicate that one border sequence is sufficient for VirD action if other repeat sequences are present.

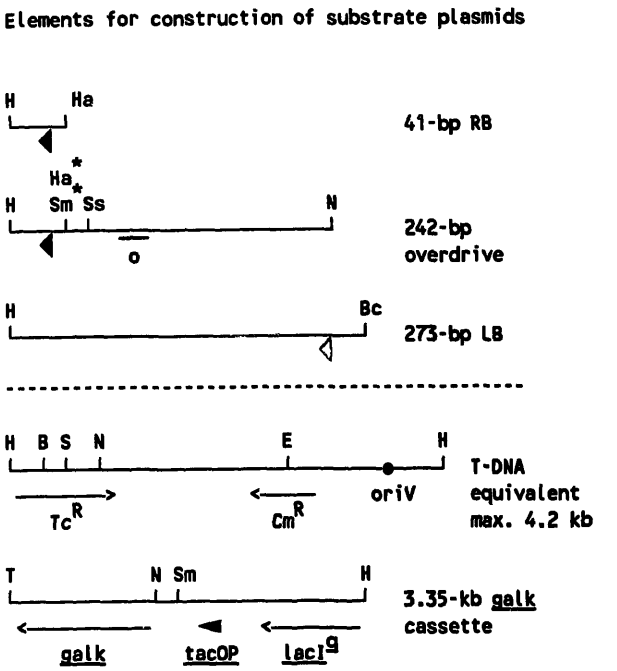


Fig. 1. Elements for construction of substrate plasmids. **RB**: a 41-bp *Hin*dIII-*Hae*III fragment was excised from pER25 (W. Ream, unpublished). It carries a synthetic RB of $T_L$ from octopine Ti plasmid pTi15955 (Barker et al., 1983). **Overdrive**: a 187-bp *Sst*I-*Nru*I fragment from pT502.1 (Alt-Mörbe et al., 1986) was cloned into vector plasmid pKC86 (Chak and James, 1985). Taking advantage from vector restriction sites, a 242-bp *Hin*dIII-*Nru*I RB-overdrive cassette was available where the natural distance between RB and overdrive was enlarged by 14 bp between the *Sma*I and *Sst*I sites from the pKC86 polylinker region. **LB**: a 267-bp *Bcl*I-*Cla*I fragment from pT502.1 (Alt-Mörbe et al., 1986) was cloned into pBR325 (Prentki et al., 1981) and excised as a *Bcl*I-*Hin*dIII fragment enlarged by 6 bp. **T-DNA equivalent**: full-length or part of vector plasmid pACYC184 (Chang and Cohen, 1978). The other elements described here were introduced into different restriction sites within the $Tc^R$ locus of this vector (see Fig. 2). ***galK*-cassette**: the 41-bp polylinker region in pFD105 (Alt-Mörbe et al., 1986) was deleted using *Sma*I and religated to give plasmid pT106. The *galK* cassette can be excised as a 3.35-kb *Hin*dIII-*Tth*111I fragment. **Symbols**: ◂, RB of $T_L$ of the Ti plasmid 15955 (Barker et al., 1983); ○, overdrive sequence (Peralta et al., 1986); ◃, LB of the right T-DNA portion of pTi15955; *oriV*, replicon; *tacOP*, *tac* promoter/operator region; *lacI*$^q$, up mutation of *lacI* repressor gene; *galk*, *galK*. Restriction sites: B, *Bam*HI; Bc, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; Ha, *Hae*III; N, *Nru*I; S, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; T, *Tth*111I; asterisk, restriction site lost during construction.

pS132.2 does not contain repeat sequences except those in RB and LB, and the relative positions of RB and LB are inverted with respect to the replicon ( = T-DNA equivalent) in comparison to the Ti plasmid. A very low background rate ($5 \times 10^{-8}$) was obtained, and *virD* enhanced T-circle production 200-fold. These results match those with substrate plasmid pT502.1 (Alt-Mörbe et al., 1986) with 'correctly' placed border sequences. Whereas in that case an LB

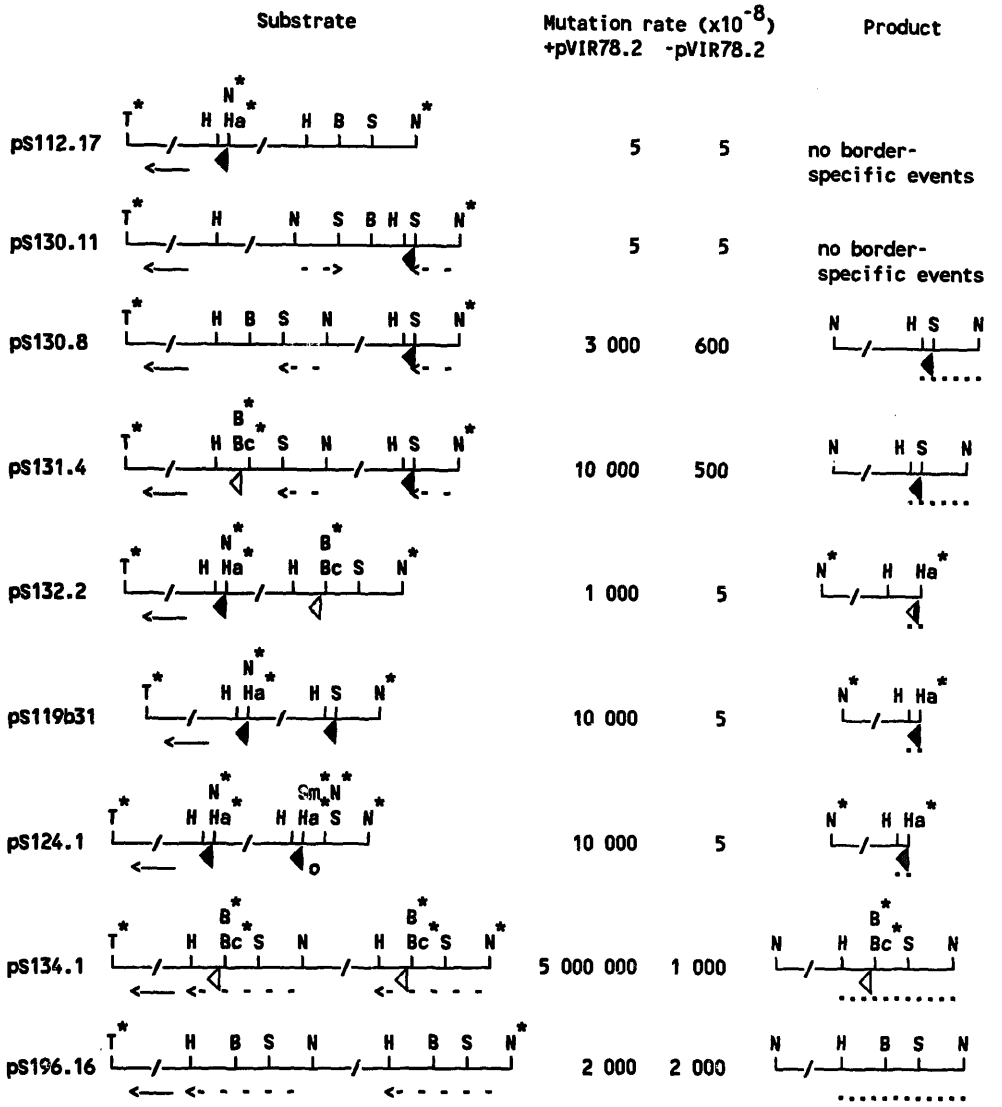


Fig. 2. T-circle formation with different substrate plasmids in the presence and absence of virD functions (pVIR78.2: Alt-Mörbe et al., 1986): efficiency and analysis of product plasmids. All substrate plasmids were constructed from the five elements summarized and described in Fig. 1. **pS112.17**: the RB element was ligated to *Hin*dIII digested pT106, followed by a cut with *Tth*111I and filled-in with PolIk. The 3.4-kb fragment was ligated into *Nru*I-digested pACYC184. **pS119b31**: pS112.17 was linearized with *Sal*I, filled-in with PolIk and partially digested with *Hin*dIII. The 621-bp *Hin*dIII-*Sal*I fragment from the Tc$^R$ locus was removed, and the larger fragment was ligated to the RB element (the *Sal*I site at the *Hae*III-*Sal*I junction is restored). Overdrive plasmid **pS124.1** was constructed similarly: instead of a second RB element, the overdrive element was ligated into pS112.17 prepared as above (*Sal*I site at the junction is restored). **pS130.8** and **pS130.11**: the gel-purified 3.7-kb *Hin*dIII fragment from pS119b31 with *galK* cassette and RB element spaced by 322 bp between *Sal*I and *Nru*I site of the Tc$^R$ locus was ligated into pACYC184 to give pS130.11 (same orientation of Tc$^R$ locus and *galK*) and pS130.8 (divergent orientation). **pS131.4**: pS130.8 was completely digested with *Bam*HI and partially with *Hin*dIII. The fragment without the 346-bp segment of the Tc$^R$ locus between these two restriction sites was ligated with the 273-bp *Bcl*I-*Hin*dIII LB-element. **pS132.2**: was constructed in the same manner from pS112.17. **pS134.1**: the LB element was cloned into *Hin*dIII + *Bam*HI-digested pACYC184; this plasmid was linearized with *Hin*dIII and ligated with the 4.2-kb *Hin*dIII fragment from pS132.2 which contains the *galK* cassette, 621 bp between *Nru*I and *Bam*HI site from the Tc$^R$ locus and one LB element. In the resulting plasmid, the *galK* cassette is flanked by two LBs embedded in a 869-bp direct repeat. **pS196.16**: the 4.3-kb *galK* containing *Sal*I fragment from pS130.8 was ligated into *Sal*I-digested pACYC184, to give a border sequence-free control plasmid, where the *galK* cassette is flanked by the 945-bp *Hin*dIII-*Nru*I direct repeat from the Tc$^R$ locus. The frequency of T-circle formation was determined with the test system described in Alt-Mörbe et al., 1986. Symbols as in Fig. 1; in addition: arrow, Tc$^R$ locus; dashed arrows, direct repeats from vector plasmid pACYC184; dotted line, sequence in which the junction occurred.

was found at the junction of the processing product, the product from pS132.2 contained an RB sequence, indicating that the junction occurred within the first 13 bp (identical in RB and LB). This detection of the RB indicates that the border at the left end of the T-DNA equivalent is retained, regardless of whether it is LB or RB. This functional equivalence of RB and LB in T-circle formation is in contrast to the polarity of T-DNA transfer into plants:

Shaw et al. (1984) and Wang et al. (1984) demonstrated that the RB is essential for transfer, but not the LB. Assuming a replicative step during processing of the transport form of T-DNA this suggests that unidirectional replication commences specifically at RB. If this process were involved in formation of T-circles, VirD would be expected to enhance elimination of *galK* from pT502.1, but not from pS132.2. Equivalent mutation rates from pT502.1 and pS132.2 therefore suggest that VirD either triggers replication at similar rates from RB and from LB or that the T-circles are formed by recombination or by ligation of a linear ds T-DNA molecule. If T-circles are related to the transport form, additional factors (others than VirD1/VirD2) are responsible for polarity in DNA transfer in the natural system.

Plasmid pS119b31 contains two synthetic RBs on both sides of the replicon unit as the only Ti-plasmid-derived sequences. The results show that no additional Ti plasmid sequences are required for high frequency of T-circle formation ($1 \times 10^{-4}$). This is confirmed with pS124.1. Although it contains in addition the 'overdrive' sequence which is described as an enhancer for plant transformation (Peralta et al., 1986) next to the right RB copy, the T-region equivalent is not processed at higher efficiency. This might, however, be due to the absence of other Vir functions.

The highest mutation rate ($5 \times 10^{-2}$) was observed with plasmid pS134.1, in which the T-DNA equivalent is flanked by two LB elements embedded in 869-bp direct repeats of vector sequences. This long duplication appears to be the reason for the high background rate without *virD*, as shown by the result with pS196.16 which carries a comparable duplication without border elements. It should be stressed that VirD increases the mutation rates only with pS134.1, not with pS196.16, indicating that the interaction of Vir functions with border sequences is necessary for the high figures obtained in these experiments.

These results show that (*i*) VirD action requires at least one border copy; one border is, however, only sufficient if other direct repeats occur on both sides of the T-DNA equivalent, and (*ii*) the left and the right copy of the border are functionally equivalent in T-circle formation.

### (b) T-DNA processing products in different *rec*$^-$ hosts of *Escherichia coli*

The results discussed above also indicate that long direct repeats on both sides of the T-DNA stimulate T-circle formation, and that in these cases the junction occurs within the large direct repeat rather than within the T-region border (pS130.8 and pS131.4). These two points argue against a simple cut-and-paste model with ds cleavage and subsequent religation, but for involvement of recombination events. We therefore examined the role of chromosomally encoded Rec functions in this process. *E. coli* is an advantageous organism for these studies: *Agrobacterium*-specific functions (except VirD) are excluded, and a good collection of different *rec* mutants is available. Homologous genes involved in recombination have been identified in different families of bacteria (Smith, 1988), and recently, Farrand et al. (1989) demonstrated that the *recA* gene from *A. tumefaciens* C58 is able to complement a *recA* mutation of *E. coli*. Therefore it is likely that recombination processes in both bacteria species are comparable. The results of these experiments are summarized in Table I. The pVIR plasmid in these experiments was pVIR78.2, not pVIR97.89 (Alt-Mörbe et al., 1986) which produces higher levels of both VirD proteins (W.H., unpublished results); however, the latter plasmid was lethal to most of the *rec* strains. It seems reasonable to assume that this is due to the impaired ability of these strains to repair cuts in the chromosome which occur in sequences similar to T-region borders.

TABLE I

Processing products from substrate plasmid pS119b31 in the presence of pVIR78.2 in various *rec* mutants of *Escherichia coli*

| *E. coli* strain | Relevant mutations | Relevant property (phenotype) | ss products | Circular ds products[b] |
|---|---|---|---|---|
| R434 | *rec*$^+$ (wt) | Rec$^+$ | none | $1–5 \times 10^{-2}$ |
| WA252 | *recA*1 *recB*21 | Rec$^-$ | none | $2 \times 10^{-4}$ |
| WA254 | *recA*56 *recB*21 *recC*22 *sbcA*23 | RecE pathway active | ssDNA | $1 \times 10^{-2}$ |
| WA255 | *recB*21 *recC*22 *sbcA*23 | RecE pathway active | ssDNA | $1 \times 10^{-2}$ |
| WA293 | *recB*21 *recC*22 *sbcB*15 | RecF pathway active | RNA[a], no ssDNA | $3 \times 10^{-1}$ |

[a] Not dependent on the presence of pVIR78.2.

[b] Determined with a modified selection system: different strains of *E. coli* were cotransformed with one VIR and one substrate plasmid, double transformants were selected during growth in liquid tryptone medium (1% Bacto tryptone/0.5% NaCl/10 mM Tris·HCl pH 7.2/1 mM $MgSO_4$/0.2% glycerol) for 24–28 h at 28°C to an absorbance of $A_{600}$ = 0.5–1. Portions of 1 $A_{600}$ were harvested for miniscale plasmid isolation, and 0.5 μg plasmid DNA were transformed into 100 μl of *E. coli* R434(*Δgal*) prepared by the $CaCl_2$ procedure (Maniatis et al., 1982). Different dilutions were then plated on nonselective and on *galK* counterselective agar and the mutation rates were determined as described (Alt-Mörbe et al., 1986).

*(1) Formation of T-circles*

In the recombination-deficient mutants WA252(*recAB*) and HB101(*recA*, data not shown), T-circle formation is drastically reduced when compared with *rec*$^+$ wt R434. In *rec*$^+$ wt cells, the RecBCD pathway is active (reviewed by Smith, 1988), i.e., the RecA protein mediates strand transfer between homologous regions of one at least partially ss region and another ds DNA molecule; for one of the molecules a free 3′ end is required. The ss molecules in wt cells are produced from ds linear molecules by the unwinding activity of the RecBCD-encoded helicase. For T-circle formation by the *recBCD* pathway of recombination, VirD1/VirD2 action might provide either ss substrate by a replicative mechanism or by helicase activity, or it might produce linear ds molecules (cleaved within one border) as substrate for the RecBCD enzyme for RecA mediated strand exchange.

With the *recBC* mutant WA255 and also with WA254 (in addition *recA*), almost the same frequency of T-circle formation is observed as in wt R434. In both strains the RecE pathway is induced by the *sbcA* mutation (Symington et al., 1985; Abastado et al., 1987). In these mutants, exonuclease VIII (the product of *recE*) is active, which creates long 3′ overhangs by digestion of one strand of a linear ds molecule starting at its 5′ end. Once the 5′ to 3′ degradation exposes regions of homology, they anneal in a snail ($\sigma$) structure from substrates with direct repeats. According to this pathway for intramolecular recombination, the optimal substrates provided by VirD would be ds substrate plasmids linearized by a cut in one border (see Abastado et al., 1987) or ss Ti plasmid equivalents. T-strands, or ds T-regions excised by two cuts should be no substrates, because they lack homologous sequences within one molecule.

Interestingly, both WA254 and WA255 produced two classes of ds product plasmids: (*1*) 3.4 kb; these correspond to the expected, correctly processed T-DNA equivalents, and (*2*) 2.6 kb; these smaller products contain *oriV* and the complete Cm$^R$ locus from pACYC184. Aberrant processing products with pACYC184 derived plasmids were also reported by De Vos and Zambryski (1989), and the most likely explanation is the presence of sequence elements in this vector which are similar to border sequences. Aberrant circularization products were also observed by Timmermann et al. (1988) and explained by recombination within repeats other than border sequences.

The *E. coli* strain with the highest T-circle production (all in the expected size) was WA293 which is mutant in *recB*, *recC*, *sbcB* and also *xth*. This strain is deficient in the *sbcB* and the *sbcB* mutation activates the RecF pathway of recombination (Smith, 1988). Both nucleases hydrolyse one strand of linear dsDNA molecules from 3′ to 5′, but circular ds molecules are unaffected. The *recBC* mutation improves the stability of ds linear molecules which are substrates for this recombination pathway. The high yield of correctly processed T-circles ($3 \times 10^{-1} = 30\%$) strongly supports a hypothesis that the VirD functions provide linear ds molecules which are cleaved within on border as substrates for recombination into T-circles.

*(2) Formation of ss products*

The ss T-DNA-related forms are shown on the autoradiogram of a native blot hybridized with a nick-translated probe from the central part of our T-DNA equivalent. All bands detectable in Fig. 3 were sensitive to S1 nuclease

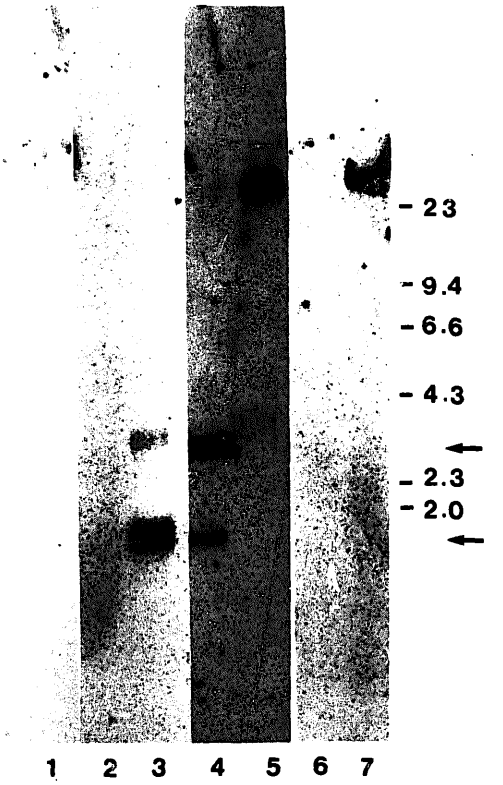


Fig. 3. Detection of ss T-DNA products on a native Southern blot. Plasmids pVIR78.2 and pS119b31 were transformed into the strains specified in Table I, and double transformants were grown for 24–30 h at 28°C. Total DNA was prepared according to Dhaese et al. (1979), electrophoresed through 0.5% agarose gels in 1 × TBE buffer (Maniatis et al., 1982), and the gel was blotted according to Albright et al. (1987) without previous alkali treatment. Probe for detection of altered or unaltered substrate plasmid was a gel purified 790-bp *Taq*I fragment from the Cm$^R$ locus within the T-DNA equivalent. Sizes of phage $\lambda$ DNA *Hin*dIII fragments are given in kb on the right margin. For another size comparison, small aliquots of substrate and product plasmid were boiled for 2 min in 30% DMSO/2 mM EDTA, cooled on ice and co-electrophoresed (arrows). Lanes: **1**, R434[pVIR78.2; pS119b31]; **2**, WA252[pVIR78.2; pS119b31]; **3**, WA255[pVIR78.2; pS119b31]; **4**, WA254[pVIR78.2; pS119b31]; **5**, WA293[pVIR78.2; pS119b31]; **6**, as lane **5**, but treated with 1 $\mu$g RNase for 20 min prior to electrophoresis; **7**, WA293[pS119b31].

(conditions: see Stachel et al., 1986; data not shown), and this confirms their ss nature.

From R434 and WA252 (Fig. 3, lanes 1 and 2) no ss products were observed. This is unexpected, because Jayaswal et al. (1987) found 5–10% of the T-regions processed into T-strands in *E. coli* JM109(*recA*1) over-expressing octopine VirD functions. Possible reasons for these differences are octopine functions instead of nopaline functions and different ratios of expression of *virD1* vs. *virD2*.

In contrast, with strains WA254(*recABC*, *sbcA*23; Fig. 3, lane 4) and WA255(*recBC*, *sbcA*22; Fig. 3, lane 3) two ss forms were detected, which were both dependent on VirD function (control without *virD* not shown). One possible explanation is that the function of the *recBCD* enzyme (exonuclease V) destroys ss forms in the wt strains, but that they are stabilized in the mutants (*recBCD* enzyme possesses ATP-dependent exonuclease activity for ss and ds DNA and also ss DNA endonuclease activity; reviewed by Smith, 1988). The ss molecules could either arise directly from *virD* action or from processing of border-cleaved linear ds molecules by the *recE* enzyme (exonuclease VIII). In the *Agrobacterium* system, ss molecules are likely to be protected by the ss DNA binding *virE2* product (Citovsky et al., 1988).

Hybridization with the T-DNA probe revealed two ss bands from WA255 and WA254. As judged from their comigration with denatured substrate and product plasmids, the upper band corresponds to the full-length ss substrate plasmid, the lower band to the ss T-DNA equivalent. An ss band corresponding to only the *galK* cassette may have been present, but would not be detected, because the hybridizing probe covered only the T-DNA equivalent. This is consistent with data by Stachel et al. (1987) with octopine Ti plasmid $pTiA_6$ which contains two T-DNA regions and four border sequences. These authors detected also ss molecules which correspond to the intervals between two or three borders. No ss DNA corresponding to the aberrant small T-circles were found which were observed in these strains. Therefore it is unlikely that T-circles are precursors of T-strands: in this case aberrant T-strands would be expected to be processed from aberrant T-circles.

The processing product of pS119b31 detected in WA293 is surprising for several reasons: it is much larger than expected for ss substrate plasmid or T-DNA, it migrates at the position of a 23-kb ds DNA (Fig. 3, lane 5), it is not dependent on the presence of *virD* (lane 7), and it is not only sensitive to S1 nuclease, but also to RNase (lane 6). Niwa et al. (1988) have recently presented evidence that border sequences (especially RB sequences) are recognized by RNA polymerase in *Agrobacterium* as well as in *E. coli* and that they function as promoter, when placed upstream of a promoterless *lacZ* gene. The fact that we found this RNA only in *E. coli* WA293 could be due to improved stability of such RNA in this special genetic background. The size, however, is astonishing: it implies that the RNA spans several circles of substrate (or product) plasmid and that RNA polymerase does not stop at terminator sequences on the template.

**(c) General conclusions**

(*1*) T-circle formation in *E. coli* is dependent on VirD1/VirD2 functions and T-region border sequences, and RB and LB are functionally equivalent. The results also indicate that these Vir functions initiate recombination processes by producing a cut within one border; the linearized plasmid molecule could serve as substrate for three recombination pathways (RecBCD, RecE, RecF). The stimulation of T-circle formation by direct repeats can be explained by the function of *recA* in combination with the activity of the *recBCD* system. It appears likely that the formation of T-circles and of T-strands is not related, because in some cases aberrant T-circles are detected, but no aberrant T-strands (*E. coli* mutants WA254 and WA255).

(*2*) Given the similarity of Rec functions in bacteria, the T-circles detected in *Agrobacterium* are likely to be produced by the same mechanisms. This would imply that the cells also contain circularized forms of the rest of the Ti plasmid; although no longer virulent, these would still contain the functions for opine utilization and conjugal transfer. T-circles are not likely to be a major transport form or its obligate precursor, because (*i*) a *recA* mutation in *Agrobacterium* does not affect virulence on plants (Farrand et al., 1989), and (*ii*) T-DNA transfer into plant cells is polar, beginning at RB (Shaw et al., 1984; Wang et al., 1984). Circularized T-regions might, however, serve as short-term storage forms which are less sensitive to nucleases than linear molecules (VirD2 protein bound to the 5′ end does not protect against 3′ → 5′ degradation; see Dürrenberger et al., 1989).

ACKNOWLEDGEMENTS

We thank W. Ream for providing plasmid pER25, W. Wackernagel for *E. coli* strains WA252, WA254, WA255 and WA293, and G. Lurz for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 206).

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