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Overdrive is a T-region transfer enhancer which stimulates T-strand production in *Agrobacterium tumefaciens*

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SUMMARY:

Introduction of a left or right synthetic border repeat together with the overdrive sequence in an octopine Ti-plasmid deletion mutant, lacking the right border, resulted in the complete restoration of the oncogenicity of the mutant strain. However introduction of a border repeat without the overdrive, only restored oncogenicity partially.

The overdrive sequence turned out to be able to stimulate the synthetic border mediated T-region transfer, independent of its orientation and position relative to the border repeat. Furthermore the distance between border repeat and overdrive could be enlarged, without a loss of overdrive activity. Here we enlarged the distance between the two sequences up to 6714bp. These results were confirmed by estimating the amount of single stranded T-DNA molecules from induced agrobacteria, containing the various border constructs.

INTRODUCTION:

By transferring part of its own DNA to the plant genome Agrobacterium tumefaciens can induce the plant disease Crown Gall on dicotyledonous plants(1,2). The transferred piece of DNA, called T-DNA, is part of the large tumour-inducing (Ti-)plasmid which is present in virulent agrobacteria. The T-region contains genes that are expressed in the plant cells. Some of these genes are onc-genes coding for enzymes involved in the production of the phytohormones cytokinin and auxin in the transformed plant cells(3,4,5). Other T-DNA genes determine the formation of certain specific compounds called opines in the tumour cells(6,7).

None of the genes present in the T-region are involved in the transfer of the T-region to the plant cell(8,9). The genes essential for T-DNA transfer are located in another region of the

Ti-plasmid called the virulence (vir) region. This region consists of seven complementation groups(VirA to VirG) that are directly or indirectly involved in the DNA-transfer process(10,11). Most of the vir-genes are silent unless they become induced via certain plant phenolics such as acetosyringone(12). The T-region is flanked by imperfect 24bp direct repeats(13). Proteins determined by the VirD operon recognize these 24bp repeats and produce nicks at one specific site in the bottom strand of each of these repeats(14). Thereafter single stranded DNA-molecules representing the bottom strand of the T-region(T-strands) are formed in the bacterium prior to DNA-transfer to the plant cell(15).

Previous work had already shown that deletion of the right border 24bp repeat resulted in an almost complete loss of oncogenicity(16,17,18), whereas deletion of the left border 24bp repeat did not lead to avirulence(8,19). A right border repeat can only mediate transfer of the onc-genes efficiently if it is present in one particular orientation relative to these onc-genes(16,20,21). Therefore it is likely that T-DNA transfer is an orientated process starting from the essential right border repeat and terminating at the left border repeat. When the left border repeat is not present, termination probably takes place at a more or less homologous sequence, a so called pseudo border sequence.

The right border 24bp repeat can be substituted by a 24bp left border repeat without a loss of virulence(21). Apparently, left and right border repeats are functionally equivalent. However, next to the right border repeat a cis-active sequence is present, which is necessary for wild-type virulence(20,21). This sequence, which was called "overdrive" by Peralta et al. and defined as a particular 24bp sequence is present immediately on the right hand side of the right border repeat of the octopine Ti TL-region(20). A similar sequence is present next to the right border repeat of the octopine Ti TR-region and also -albeit with less homology- next to the nopaline Ti T-region and the agropine Ri TL-region right border repeats(17,22,23). The overdrive sequence by itself cannot mediate T-DNA transfer to the plant cell(20,21). This implies that a 24bp repeat is essential in the transfer process,

but not enough to allow wild-type levels of transformation.

In this article we demonstrate that the overdrive can stimulate T-region transfer to the plant cell from different positions relative to a synthetic 24bp right border repeat and that it is involved in the production of single stranded molecules in acetosyringone induced bacteria.

Materials and methods:

Bacterial strains

Escherichia coli strain JM101(*lac*, *pro*AB, *sup*E, *thi*, F' D36, *pro*AB$^+$, *lac*Iqz$^+$, M15) was used in selection experiments for fragments inserted into pIC-vectors(24,25). The *E.coli* strain KMBL1164(*thi*, *pro*, *lac*; P. van de Putte) was used as a background in conjugation experiments(18) and plasmid pRK2013(Km, Tra+ RK2), was used to mobilise the non-conjugative plasmids in these conjugation experiments(26).

A.tumefaciens strains LBA1010(Rif; pTiB6) and LBA288(Rif; no Ti-plasmid) were used as a positive and negative control in the virulence assay experiments, respectively(27,28). Strain LBA4417(Rif, pTiAch5, Sm, *ocs*, *ape*, *occ*) is the mutant strain used in this study(18).

DNA isolation procedures

DNA from *E.coli* strains was isolated by the procedure of Birnboim and Doly(29). DNA from *A.tumefaciens* was isolated as described by Ooms *et al.*(30).

Construction of recombinant DNA plasmids

The techniques used for the construction of the different border clones are all described in Maniatis *et al.*(31). The border fragments were chemically synthezised(courtesy of Dr.H.De Boer, Genentech Inc, San Fransisco) and cloned as a SalI-HindIII fragment(right border repeat; octopine TL-region) or a SstI-XbaI fragment(left border repeat; octopine TL-region) in both pIC19R and pIC20R(25), and from there into the EcoRI-site of the shuttle vector pRAL5200 described previously(21).

The two enhancer fragments were also both initially cloned into pIC-cloning vectors: the 626bp overdrive containing fragment as a SstI-BclI fragment(coordinates 14087-14713) and the 189bp

overdrive containing fragment as a SstI-NruI fragment(coordinates 14087-14276).

With the above mentioned subclones we were able to construct the various clones discussed in this article. For instance the 189bp fragment was cloned in two orientations on both sides of the synthetic right border(cloned in pIC19R) in the unique NruI- and SmaI-site, respectively, to obtain the constructs pRAL5265, pRAL5267, pRAL5269 and pRAL5271.

The unique HindIII-site available in pRAL5265, between border repeat and overdrive, was used to construct plasmid pRAL5303 in which the distance between border repeat and overdrive is enlarged by insertion of a 1.5Kb HindIII-fragment containing the neomycin phosphotransferase gene(32). Similary in plasmids of the series pRAL5304-pRAL5307 different fragments of the phage lambda genome(33) were inserted into this HindIII-site.

Fragments containing synthetic border repeats were sequenced in pRAL5200, using primers hybridising directly left and right of the unique EcoRI site in pRAL5200. Sequence reactions were performed as described by Chen and Seeburg(34).

Introduction of the border fragments in LBA4417

After insertion of the Ti-fragments into pRAL5200(21), the resulting plasmids were mobilized to LBA4417 in a tri-parental mating with the helper plasmid pRK2013. Transconjugants were selected on their rifampicin(20ug/ml) and spectinomycin(250ug/ml) resistance by plating on mineral medium with glucose(35).

Insertion of the shuttle vector in pAL4417 at the proper place and orientation was verified by Southern blot hybridisation experiments. Total DNA isolates of the A.tumefaciens strains were cut with BamHI or EcoRV and after blotting hybridized with labeled pRAL5200(data not shown).

The number of inserted shuttle vectors was checked by probing a Southern blot of EcoRI digested total DNA with pBR322(data not shown). In the experiments presented here constructs were used with only a single insert of the loaded shuttle vector in pAL4417, although we could not see any difference in tumorigenicity between strains containing a single insert and strains with multiple inserts of the loaded shuttle vector.

Isolation of single stranded T-region molecules

Bacteria were induced with acetosyringone(100 ug/ml) for 18 hours, after which total DNA was isolated from them. The untreated DNA(2ug) was loaded on a 0.6% Tris Borate EDTA buffer agarose gel. After electrophoresis the nondenatured DNA was transferred to a nitrocellulose membrane and hybridized with a probe specific for the T-region; i.e. a RsaI fragment(coordinates 8497-9836) containing the cytokinin gene.

Virulence assays

For each construct two independent transconjugants were tested at least twice on stems of Nicotiana glauca, Kalanchoe daigremontiana, Kalanchoe tubiflora and Lycopersicon esculentum, as described previously(11). Tumour formation was scored three weeks after inoculation of the plants with A.tumefaciens.

RESULTS:

Changing the position of the overdrive relative to the synthetic right border repeat.

The non-oncogenic mutant strain LBA4417 carries the deleted octopine Ti-plasmid pAL4417, which lacks the entire TR-region and

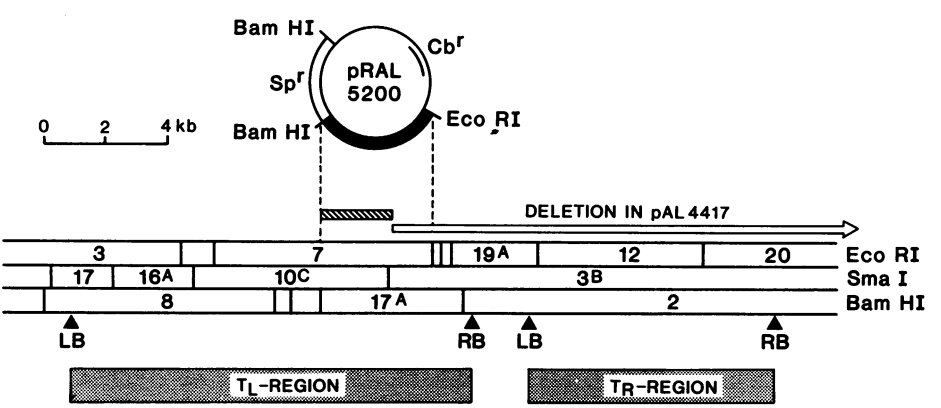

Figure 1: Restriction endonuclease map of part of the octopine TL-region. The homology between the Ti-plasmid and the shuttle vector, pRAL5200, is indicated with the hatched bar. Between these homologous regions a single cross over event can take place, resulting in a new strain harbouring a Ti-plasmid with the shuttle vector inserted into it. The unique EcoRI-site in pRAL5200 was used for the insertion of border fragments.

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   SstI        24bp Overdrive sequence          NruI          BclI
E:GAGCTCGTGTGAATAAGTCGCTGTGTATGTTTGTTTG-152bp-TCGCGA-431bp-TGATCA
N:GAGCTCGTGTGAATAAGTCGCTGTGTATGTTTGTTTG-152bp-TCGCGA
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Figure 2: The 626bp(E) and 189bp(N) overdrive fragment used in this study. The sequence is derived from Barker et al.(21).

the right border of the TL-region. With the help of shuttle vector pRAL5200 we introduced DNA fragments cloned from the wild-type Ti-plasmid into pAL4417 in order to see whether they could replace the deleted right border(Fig.1;21). Virulence was fully restored only when the introduced fragments comprised not only the right border 24bp repeat but also neighbouring sequences. In this way it was found that rightward from the right border repeat there is a sequence present, which is necessary for wild-type virulence. We found that this sequence, which is called the "overdrive"(20) is present in a 626bp SstI-BclI fragment(called E throughout this paper) or a 189bp SstI-NruI fragment(called N; Fig.2). Enhancers of transcription and recombination work irrespective of their position and orientation versus the transcriptional startsite and repeat sequence, respectively. In order to find out whether the "overdrive" shares these characteristics with enhancers we varied the position of this sequence versus the 24bp border repeat in the experiments described below.

In order to address this question we first cloned a 189bp fragment(N) containing the overdrive on the right side of a synthetic right border repeat(SRB) in both orientations. Introduction of these fragments via pRAL5200 into pAL4417 resulted in strains LBA5265(overdrive in normal orientation) and LBA5267(overdrive in inverted orientation). Virulence tests on Nicotiana glauca, Lycopersicon esculentum(tomato), Kalanchoe daigremontiana and K. tubiflora revealed that not only LBA5265 had wild-type tumorigenicity as expected, but the same was the case for LBA5267(Fig.3). This shows that the overdrive is functional in both orientations versus the 24bp border repeat. Next we tested whether the overdrive could be active also when inserted on the other side of the 24bp repeat thus from a

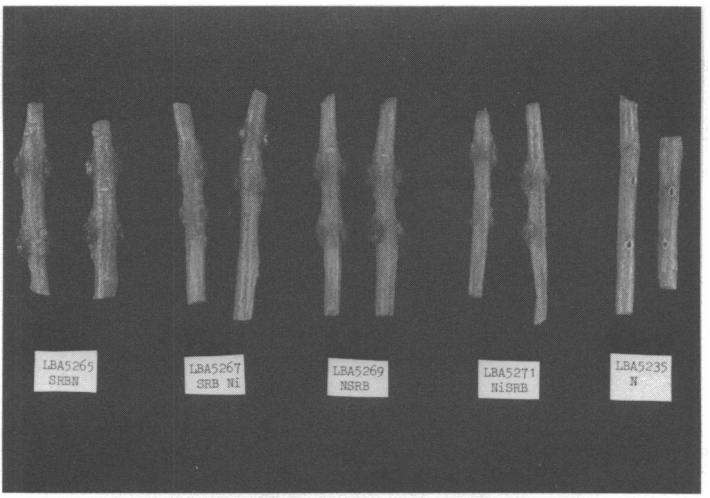

Figure 3: Inoculation tests on stems of L.esculentum of the strains LBA5265(SRBN), LBA5267(SRB N inverted), LBA5269(NSRB) and LBA5271(N inverted SRB).
Abbreviations stand for: SRB=synthetic right border repeat and N=189bp overdrive fragment.

position within the T-region. To investigate this the strains LBA5269(overdrive inside; normal orientation) and LBA5271(overdrive inside; inverted orientation) were constructed. These strains were equally virulent as the wild-type on the plant species mentioned above(Fig.3). This demonstrates that the overdrive is not only active in both orientations, but also irrespective of whether it is at a position leftward or rightward from the 24bp repeat.

Chanching the distance between overdrive and border repeat

In the wild-type Ti-plasmid the spacing between overdrive and the 24bp border repeat is only 14bp(TL-region) or 13bp(TR-region)(20). In our experiments however, the distance between overdrive and 24bp border repeats varied considerably(up to 163bp to the right of the repeat in LBA5267 and up to 156bp to the left of the repeat in LBA5269). Apparently this does not interfere with its activity. To test whether this distance could be enlarged even more, we inserted different DNA fragments between the overdrive and the border sequence in the HindIII-site of pAL5265. Insertion of a 1500bp fragment containing the neomycin

Table I. Oncogenicity assays. Plants were scored 3 weeks after inoculation. Each strain was tested at least twice on every test plant.

Strain	Border fragment	Spacing	Oncogenicity assays			
			L.esc	K.dai	K.tubi	N.glauca
LBA1010(pTiB6)		14	+++	+++	+++	+++
LBA4417(pAL4417)		-	-	-	-	-
LBA5200(pAL5200)	-(shuttle vector)	-	-	-	-	-
LBA5209(pAL5209)	E	-	-	-	-	-
LBA5235(pAL5235)	N	-	-	-	-	-
LBA5251(pAL5251)	SRB	-	+/-	+	+	+
LBA5253(pAL5253)	SRBE	42	+++	+++	+++	+++
LBA5255(pAL5255)	SLB	-	+/-	+	+	+
LBA5257(pAL5257)	SLBE	39	+++	+++	+++	+++
LBA5261(pAL5261)	E-inverted SRB	33	+++	+++	+++	+++
LBA5264(pAL5264)	E-inverted SLB	35	+++	+++	+++	+++
LBA5265(pAL5265)	SRBN	32	+++	+++	+++	+++
LBA5267(pAL5267)	SRB N-inverted	163	+++	+++	+++	+++
LBA5269(pAL5269)	NSRB	156	+++	+++	+++	+++
LBA5271(pAL5271)	N-inverted SRB	25	+++	+++	+++	+++
LBA5303(pAL5303)	SRB neo N	1433	+++	+++	+++	+++
LBA5304(pAL5304)	SRB 2.0 N	2059	++	+++	+++	+++
LBA5305(pAL5305)	SRB 2.3 N	2354	++	+++	+++	+++
LBA5306(pAL5306)	SRB 4.3 N	4381	++	++	+++	+++
LBA5307(pAL5307)	SRB 6.7 N	6714	++	++	+++	+++
LBA5304a(pAL5304a)	SRB 2.0	-	+/-	+/-	+	+
LBA5305a(pAL5305a)	SRB 2.3	-	+/-	+	+	+
LBA5306a(pAL5306a)	SRB 4.3	-	+/-	+/-	+	+
LBA5307a(pAL5307a)	SRB 6.7	-	+/-	+	+	+

abbreviations stand for: LB=left border; SRB=synthetic right border; SLB=synthetic left border; E=626bp enhancer fragment; N=189bp enhancer fragment; TL=TL-region of the octopine Ti-plasmid; neo=neomycin phosphotransferase(ca. 1.4 kbp); 2.0, 2.3, 4.3 and 6.7= HindIII fragments of the phage lambda genome of resp. 2.0, 2.3, 2.0 + 2.3 and 6.7 kbp.
L.esc=Lycopersicon esculentum; K.dai=Kalanchoe daigremontiana; K.tubi=Kalanchoe tubiflora; N.glauca=Nicotiana glauca.
symbols indicate: -, avirulent; +/-, weakly virulent; + to ++, partially virulent; +++, fully virulent.
spacing indicates the distance in base pairs between the 24bp border repeat and the 24bp enhancer sequence.

phosphotransferase gene between the two sequences(LBA5303) did not result in a loss of overdrive activity. Sequences of the phage lambda genome inserted into the HindIII-site of pAL5265, resulting in strains LBA5304 to LBA5307, enlarged the distance between the two sequences with 2059-6714bp. In non of these cases this led to a loss of overdrive activity(TableI). Although strains in which the distance between the two sequences became larger than 2059bp(LBA5305-LBA5307) were slightly attennuated in tumour induction on some test plants.

In order to verify whether the enhanced tumorigenicity of strains LBA5304-LBA5307 compared to strain LBA5251 is indeed due to overdrive activity and not caused by the inserted lambda fragments, we deleted the overdrive from the Ti-plasmids in these strains. The virulence of the resulting strains(LBA5304a, LBA5305a, LBA5306a and LBA5307a) was similar to that of the

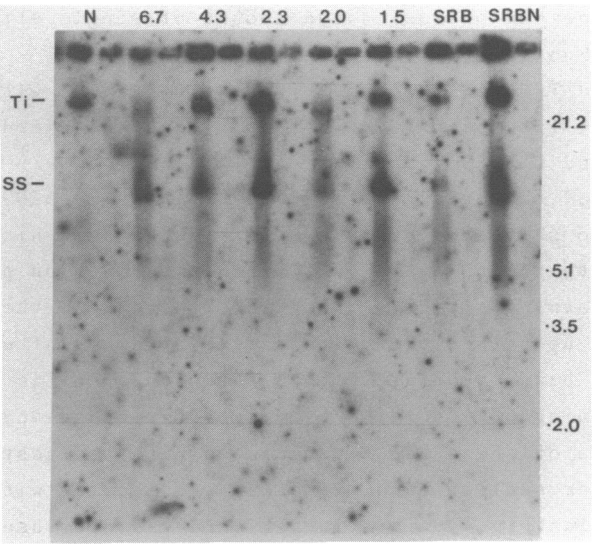

Figure 4: Analysis of total DNA prepared from acetosyringone induced and non-induced strains, LBA5235(N; lanes 1,2), LBA5307(6.7; lanes 3,4), LBA5306(4.3; lanes 5,6), LBA5305(2.3; lanes 7,8), LBA5304(2.0; lanes 9,10), LBA5303(1.5; lanes 11,12), LBA5251(SRB; lanes 13,14) and LBA5265(SRBN; lanes 15,16). Note that the slightly weaker signal in lane 9 is due to the smaller amount of total DNA loaded in this lane.
The numbers on the right side of the gel corrrespond to fragments of the lambda marker in Kbp.
Abbreviations indicate: Ti=Ti-plasmid; SS=single stranded T-DNA molecules; N=189bp overdrive fragment and SRB=synthetic right border repeat.

strain lacking the overdrive LBA5251(TableI). This shows that the inserted lambda DNA fragments themselves do not contain sequences with overdrive activity.

Single stranded molecules produced in strains containing pAL4417 derivatives:

Single stranded T-DNA molecules(T-strands) produced by Agrobacterium upon induction with a plant factor like acetosyringone are thought to be intermediate structures formed in the processing of the T-region before transfer to the plant cell. To test whether the amount of single stranded T-DNA produced after induction of the vir-region, is a measure for the tumorigenicity of Agrobacterium strains, we induced strains containing the above mentioned border constructs with

acetosyringone and estimated the amount of single stranded T-DNA produced on Southern blots.

As expected T-strands were not found in strains with Ti-plasmids that lack both right border repeat and overdrive, such as LBA4417 and LBA5200. Strains that did not contain the border repeat but had the overdrive sequence such as LBA5209 and LBA5235, also showed no production of T-strands, which is in agreement with their inability to induce tumours on plants. Strains containing a right border repeat did show the production of T-strands upon induction with acetosyringone. After Southern blotting two bands hybridized to a T-region specific probe in the lanes containing DNA isolated from acetosyringone-induced bacteria. One of these bands comigrated with undigested total DNA; the other smaller band migrated as molecules with the size of the T-region(Fig.4). This latter band was not susceptible to RNase treatment, but was susceptible to S1-nuclease treatment and only hybridized with one strand of the T-region(data not shown). Therefore it was concluded that this latter band indeed is equivalent to the band corresponding to T-strands found by Stachel et al.(15).

Strains containing a Ti-plasmid with both border repeat and overdrive produced a large amount of T-strands upon induction with acetosyringone, but strains containing a Ti-plasmid with the border repeat but lacking the overdrive showed clearly less T-strand production(Fig.4). This reveals a correlation between T-strand production and virulence. The T-strand production found in the strains LBA5303 to LBA5307(Fig.4), in which the distance between border repeat and overdrive varied, is consistent with their virulence properties.

DISCUSSION:

It is evident from this and previous publications that the presence of the overdrive in the octopine Ti-plasmid is necessary to obtain wild-type virulence(20,21).

Overdrive containing fragments do not mediate T-DNA transfer to the plant cells by themselves; a 24bp border repeat must be present in cis to start the transfer process. One of the first steps in the transfer process is the nicking of the bottom strand

of the border repeats by products encoded by the virD operon(14,15). It is not known which step in the T-DNA transfer process is stimulated by the presence of the overdrive. However, since nicks are introduced with equal efficiency in left borders(which lack the overdrive) as in right borders(which have the overdrive), it is likely that the overdrive has a positive influence on a step which follows border nicking(36,37).

In this article we show that a 189bp overdrive containing fragment can stimulate T-region transfer to the plant cell independent of its orientation and position relative to the synthetic right border repeat(Table I). By using a synthetic border sequence we excluded the role of any other sequences present in a border fragment and thus the observed results are solely due to the presence of the overdrive in the constructs.

The distance between the 24bp border repeat and the 24bp overdrive sequence varied considerably in these experiments(Table I). In a wild-type octopine Ti-plasmid the distance between the 24bp border repeat and the 24bp overdrive sequence is 14bp for the TL-region border and 13bp for the TR-region border. Here we enlarged this intervening space without losing overdrive activity. Even over distances up to 6.7Kbp the overdrive is still able to stimulate the T-region transfer process. However at distances of 2059bp or more, the stimulating effect is slightly diminished, respecting the results obtained on K.daigremontiana and L.esculentum. The strong stimulating effect of the overdrive is still clear however, when the results are directly compared to those obtained with LBA5251 and LBA5304-LBA5307, in which no overdrive is present(Table I). The stimulating effect is weakest in LBA5307, in which overdrive and border repeat are separated by 6714bp. In LBA5235 in which overdrive and border repeat are 13Kbp apart, no transfer of the T-region can be detected, although an inverted border repeat is able to mediate T-region transfer when the overdrive is nearby(21). Therefore it can be expected that the distance over which the overdrive can stimulate a border mediated transfer process efficiently will not exceed the 13Kbp. The inverse correlation between overdrive activity and the distance between border repeat and overdrive has also been reported for the regulatory sequences that control the

transcription initiation of the deo-operon in E.coli. Here the degree of repression of the operon is inversely related to the distance between the operator sites(38).

The production of single stranded T-DNA molecules by acetosyringone-induced bacteria are in line with the ability of these bacteria to induce a tumourous respons on the test plants. Strains that are unable to induce tumour formation did not produce T-strands upon induction. However, all strains that have a left or right border repeat inserted into pAL4417 produce T-strands upon induction with acetosyringone. The amount of T-strands produced is dependent on the presence of the overdrive in the pAL4417 derivative. Strain LBA5251, which does not contain the overdrive next to the inserted synthetic right border repeat, produced a smaller amount of T-strands than the strains containing a pAL4417 derivative with both border repeat and overdrive(Fig.4). Also consistent with the results obtained in he tumour assay experiments is the slightly smaller amount of T-strands produced by the induced strains LBA5304-LBA5307(Fig.4). These results confirm the results presented by Stachel et al(15,37) and prove the involvement of the overdrive in the production of single stranded T-DNA molecules.

The extra band found in our experiments on the Southern blots in the lanes containing DNA of induced bacteria, comigrates with undigested total DNA. Transfer of T-DNA sequences to the nitrocellulose membrane under non-denaturing conditions resulting in the extra band is probably due to the nicking on the left border repeat present in all pAL4417 derivatives. The fact that this was not found by Stachel et al. and not always in our experiments(data not shown) is probably due to the procedure used to isolate the DNA from the bacteria or to different conditions during Southern blotting.

The features found here for the T-region overdrive are comparable to those of earlier reported enhancers. The recombinational enhancers of the Gin-mediated G-inversion of bacteriophage Mu(39), the Hin-mediated inversion in Salmonella(40), the Cin mediated inversion in bacteriophage P1(41) and the Pin mediated recombination in E.coli(42), the transcriptional enhancers of for instance the beta-interferon

gene(43,44) and the replication enhancer of the SV40 replicator(45), all have the ability to stimulate a certain process if inserted *in cis*, and the stimulating effect is independent on their orientation and position. Although the precise function of enhancer sequences is still unknown, like the other enhancers the overdrive probably also has a signal function in the T-region transfer process. From the fact that the overdrive is involved in the production of T-strands it can be concluded that the overdrive indeed is a T-region transfer enhancer. By forming a secondary DNA-structure after by binding a protein determined by the Vir-region or the host chromosome, this sequence may function as a signal stimulator in T-DNA processing.

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