Agrobacterium proteins VirD2 and VirE2 mediate precise integration of synthetic T-DNA complexes in mammalian cells

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***Agrobacterium tumefaciens*-mediated plant transformation, a unique example of interkingdom gene transfer, has been widely adopted for the generation of transgenic plants. *In vitro* synthesized transferred DNA (T-DNA) complexes comprising single-stranded DNA and *Agrobacterium* virulence proteins VirD2 and VirE2, essential for plant transformation, were used to stably transfect HeLa cells. Both proteins positively influenced efficiency and precision of transgene integration by increasing overall transformation rates and by promoting full-length single-copy integration events. These findings demonstrate that the virulence proteins are sufficient for the integration of a T-DNA into a eukaryotic genome in the absence of other bacterial or plant factors. Synthetic T-DNA complexes are therefore unique protein:DNA delivery vectors with potential applications in the field of mammalian transgenesis.**

Keywords: *Agrobacterium*; VirD2; VirE2; T-DNA integration; mammalian transgenesis

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INTRODUCTION

The ability to generate transgenic mammalian cells and organisms is critical for many aspects of basic and applied research. Numerous approaches such as delivery of naked (Herweijer & Wolff, 2003), chemically coated (Luo & Saltzman, 2000) or virally encapsidated (Lundstrom, 2003) DNA have been developed to facilitate this task. Ideally, a DNA delivery system should be efficient, at the same time avoiding integration of multiple transgene copies, as this frequently leads to gene silencing (Garrick *et al*, 1998). Methods relying on delivering naked or chemically coated DNA suffer from low integration rates (Wiethoff & Middaugh, 2003) and often result in multiple copies of the transgene integrated into a single locus (Pomerantz *et al*, 1983). Virus-mediated gene delivery usually results in high integration

rates and facilitates simple integration patterns; however, issues such as costs, limited transgene size and biosafety still remain (Dobbelstein, 2003). More recent approaches taken to gene delivery such as the use of the *Sleeping Beauty* transposon (Izsvak & Ivics, 2004) or bacteriophage ϕ C31 recombinase (Thyagarajan *et al*, 2001) hold much promise and may help to alleviate many of the problems described above.

Thanks to *Agrobacterium tumefaciens*, problems hampering mammalian gene delivery can be circumvented in plants. This unique plant pathogen developed a mechanism that allows it to transfer and randomly integrate a specific fragment of its tumour-inducing plasmid (pTi), the transferred DNA (T-DNA), into the genome of a plant (Zupan *et al*, 2000; Tzfira & Citovsky, 2002). In nature, the transferred genetic information is essential for pathogenesis. However, as all the genes required for production and transfer of T-DNA reside outside the T-DNA, its pathogenic sequences can be replaced, thus turning *Agrobacterium* into a powerful tool for plant transgenesis.

The bacterium's virulence proteins, VirD2 and VirE2, were shown to have key roles in the nuclear uptake and genomic integration of T-DNA in plants. The synthesis of the T-DNA from the pTi plasmid involves the cleavage of the DNA by VirD2 at the two so-called borders delineating the T-DNA. Following the cleavage, VirD2 remains covalently bound to the 5' end of the single-stranded (ss) T-DNA via a phosphotyrosine bond (Pansegrau *et al*, 1993; Jasper *et al*, 1994) and protects the DNA from exonucleolytic degradation (Dürrenberger *et al*, 1989). The NLS domain of VirD2 is important for import of the complex into plant nuclei (Howard *et al*, 1992; Tinland *et al*, 1992; Ziemienowicz *et al*, 2001), and also facilitates import of T-DNA into animal nuclei *in vitro* (Ziemienowicz *et al*, 1999). VirD2 is responsible for the efficiency of integration and preservation of the 5' ends of the integrated T-DNA (Tinland *et al*, 1995). VirE2 is a sequence nonspecific ssDNA-binding protein that covers the length of the T-DNA (Citovsky *et al*, 1997 and references therein) and maintains it in a conformation facilitating *in vitro* nuclear import (Ziemienowicz *et al*, 1999, 2001). Plant transformation using *Agrobacterium* strains deficient in VirE2 results in drastically reduced transformation efficiency and extensive deletion of the T-DNA 3' ends (Rossi *et al*, 1996). These findings imply that VirE2

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and VirD2 are not only essential for the nuclear import of T-DNA but also have a vital role in ensuring the integration of intact T-DNA units.

The integration process is thought to commence with the 3' end of the T-DNA invading the plant DNA through limited base pairing (Tinland & Hohn, 1995). The sites of integration appear to be randomly selected, and sites of DNA damage at one or both strands probably serve as T-DNA entry points (Salomon & Puchta, 1998). Following strand invasion, DNA polymerase is thought to synthesize the complementary strand of the T-DNA and remove the VirE2 molecules. *In vitro* studies of T-DNA integration showed that it is DNA ligase dependent (Ziemenowicz *et al*, 2000), suggesting that the T-DNA exploits the host DNA repair machinery. A frequent outcome of the process is a single, often full-length, copy of the T-DNA randomly integrated in the plant genome, with microhomologies between the T-DNA and host genome at both junctions (Brunaud *et al*, 2002 and references therein). Such 'clean' integration patterns usually associated with a short deletion of the host DNA are the hallmarks of T-DNA integration.

Agrobacterium-mediated transformation protocols have been successfully adopted for non-plant hosts such as *Saccharomyces cerevisiae* (Bundock & Hooykaas, 1996; Piers *et al*, 1996) and filamentous fungi (de Groot *et al*, 1998; Rolland *et al*, 2003). Transformation of HeLa cells mediated by *Agrobacterium* has also been described (Kunik *et al*, 2001), demonstrating that T-DNA integration into the animal genome is indeed possible, even if the need to use live bacteria makes this approach prohibitive for most biotechnological and medical applications.

In this work we demonstrate that synthetic T-DNA complexes integrate into the mammalian genome following transfection of cultured cells. VirD2 and VirE2 improve the efficiency of integration. The observed single-copy, full-length transgene integration patterns resemble those of T-DNAs integrated into plant genomes.

RESULTS AND DISCUSSION

T-DNA complex synthesis

A ss pre-T-DNA molecule containing the border sequence and the hygromycin resistance gene was excised from the ss phage genome using oligonucleotide-directed restriction digest (Fig 1A). ss pre-T-DNA was used as substrate for VirD2 processing (Fig 1B) and VirE2 ssDNA coating (Fig 1C). Specific assays showed each step in T-DNA synthesis to be more than 90% efficient (Fig 1B,C).

Transformation of HeLa cells with T-DNA complexes

The efficiency of integration into the HeLa genome was tested for four versions of T-DNA: naked pre-T-DNA, T-DNA processed or either by VirD2, pre-T-DNA coated with VirE2 or the complete T-DNA complex consisting of ssDNA and both proteins. Complexes were transfected into HeLa cells using cationic liposomes, and hygromycin-resistant transformants were selected. The increase in the efficiency of stable transformation was twofold with complexes containing one of the virulence proteins and nearly fourfold with the full T-DNA complex, compared with naked T-DNA (Fig 2). Although significant, this finding does not reflect the requirement shown for both proteins in nuclear import experiments (Ziemenowicz *et al*, 2001), or *Agrobacterium*-mediated transformation of plants (Howard *et al*, 1992; Tinland *et al*, 1995; Rossi *et al*, 1996). This discrepancy may be due to the fact that both transformation and nuclear import experiments

Fig 1 | T-DNA complex synthesis. (A) Cleavage of the ss pre-T-DNA molecules out of the M13 phage DNA. Oligonucleotides complementary to the *PvuII* sites flanking the pre-T-DNA region are indicated with arrows. Lane 1: Uncut m13Y3-ks-Hyg phage; lane 2: m13Y3-ks-Hyg cut with *PvuII* with the help of oligonucleotides Y3-*PvuII*3' and Y3*PvuII*5'. (B) Processing of the pre-T-DNA by VirD2 protein. Lane 1: Unprocessed pre-T-DNA (2.3 kb); lane 2: T-DNA (2.0 kb) processed by VirD2. (C) Single-strand-binding activity of the VirE2 protein.

Fig 2 | Stable transformation of HeLa cells using synthetic T-DNA complexes. Relative number of hygromycin-resistant clones selected 2 weeks after transfection. Error bars represent standard deviation from three independent experiments. Total numbers of stable clones obtained with each complex are shown.

require the passage of the T-DNA complex across the nuclear membrane. In contrast, the nuclear membrane of cultured cells is disassembled during every cell division, possibly allowing for passive uptake of naked DNA or partial T-DNA complexes. The efficiency of stable transfection of HeLa cells with a double-stranded DNA (dsDNA) version of the pre-T-DNA was similar to that obtained with ssDNA. These levels were 10- to 20-fold lower than those observed with ss and ds circular molecules carrying the hygromycin resistance marker. These findings indicate that regardless of the form of the DNA, exonucleases are the main processing activities responsible for the degradation of exogenous DNA.

Precision of T-DNA complex integration

DNA from clones stably transfected with different forms of T-DNA was monitored by Southern analysis following digestion at the unique *Sac*II site located in the polyadenylation signal of the hygromycin resistance gene, 967 nucleotides (nt) from the 3' end of the T-DNA (Fig 3). Remarkably, most hybridization patterns were consistent with the presence of just one transgene copy in the HeLa genome. The number of clones showing multiple bands upon *Sac*II digestion, consistent with a higher transgene copy number, was 1 of 10 (10%), 1 of 14 (7%), 2 of 14 (14%) and 7 of 30 (23%) for cells transfected with ssDNA, ssDNA:VirE2, ssDNA:VirD2 and ssDNA:VirE2:VirD2, respectively (see supplementary information online). This finding may indicate that the delivery of the transgene as ssDNA may itself be sufficient to lessen the likelihood of multicopy insertions. DNA from the same clones was also digested with a combination of *Sac*II and *Pvu*II restriction enzymes, which allowed the determination of the state of transgene integrity at the 5' junction, in cell lines containing single-copy insertions. The unique *Pvu*II site overlaps with the VirD2 processing site (Fig 3A) and is positioned exactly 267 nt from the 5' end of the pre-T-DNA. Following VirD2 processing, this site is located just 1 nt away from the 5' end of the T-DNA to which the VirD2 protein is attached. In the case of lines

Fig 3 | Improved fidelity of transgene delivery using T-DNA complexes. (A) Scheme of the restriction enzymes and probe used in Southern analysis. (B) Southern analysis of DNA isolated from two representative hygromycin-resistant HeLa lines transfected with the following: lanes 1–4, ssDNA; lanes 5–8, ssDNA:VirE2; lanes 9–12, ssDNA:VirD2; lanes 13–16, ssDNA:VirD2:VirE2. Restriction enzymes *Pvu*II and *Sac*II are indicated by P and S, respectively. The arrows show the 1.2-kb band indicative of the preserved *Pvu*II and *Sac*II restriction sites. The fractions and the percentages of clones (%) that showed the presence of the diagnostic 1.2-kb fragment are indicated below.

transfected with naked or VirE2-coated pre-T-DNA, preservation of both sites indicates that less than 267 and 967 nt out of the total 2380 nt were lost from the 5' and 3' ends, respectively. The frequency with which both sites were preserved is much higher for cell lines transformed with a VirE2-containing complex, as compared with cell lines transformed with naked ssDNA. This indicates that VirE2 protects the DNA from the action of endo- and exonucleolytic degradation (Fig 3B). A similar degree of DNA conservation could be observed in the cases in which VirD2 was used to process the pre-T-DNA. Even the *Pvu*II site only 1 nt away from the processing site is preserved in most cases (Fig 3B), strongly suggesting that similar to *Agrobacterium*-mediated transformation of plants (Tinland et al, 1995), the covalently attached VirD2 protein protects the 5' end from exonucleolytic activity and thus has a crucial role in maintaining transgene integrity.

Transgene integrity was further evaluated by isolating the junctions between ends of the integrated T-DNAs and HeLa genomic DNA from four additional HeLa clones stably transfected with the full T-DNA complex (Fig 4A). As only four independent

Fig 4 | Genomic junctions of integrated T-DNAs. (A) Relative positions of primers and restriction sites. (B) Properties of hygromycin-resistant transformants. Clone number, chromosomal location of inserts and the junction nucleotides are indicated. The extent of deletions incurred by the T-DNA ends and the HeLa genome is shown above and below the respective icons. Microhomologies identified between the HeLa genomic DNA and 3' ends of the T-DNA are shown where applicable.

T-DNA insertion events were analysed, general conclusions cannot be drawn. Even so, some interesting facts regarding preservation of T-DNA by virulence proteins as well as some additional, interesting parallels to previously described plant T-DNA insertion events are clearly apparent.

In all four cases, the T-DNAs were integrated into different chromosomes and all 5' junction sequences contained fully preserved sequences originating from VirD2 processing of the border (Fig 4A). Deletions in the HeLa genome concomitant with T-DNA integration spanned 2, 15, 26 and 878 base pairs (bp), whereas the 3' ends of the T-DNAs were truncated by 16, 17, 59 and 228 bp, respectively. Furthermore, for three of four integration events, microhomologies of 1–3 bp were found between the 3' ends of the T-DNAs and their respective pre-insertion sites (Fig 4B). Surprisingly, no filler DNA sequences, commonly associated with T-DNA integration in plants (Salomon & Puchta, 1998), were observed in HeLa cells. This situation may reflect the

more conservative mode of DNA repair observed in mammals (Pelczar *et al*, 2003).

The T-DNA of clone C04 was integrated into a cluster of (AATAA)_n (AT)_n repeats on chromosome 12, resulting in the deletion of almost the entire repeat block of 878 bp. Insertion C08 occurred on chromosome 11 between L1MC5 and L1ME3 repeats, whereas in line C14 the T-DNA had integrated into chromosome 4 between two MER1 repeats resulting in only limited loss of host DNA (15 and 26 bp, respectively). Finally, insertion event C10 occurred on chromosome 13, resulting in a concomitant 2 bp deletion in an L1M4c repeat. The propensity with which T-DNAs integrated into AT-rich regions is very apparent and is highly reminiscent of the situation in plants (Brunaud *et al*, 2002). The relative AT richness may favour the integration of T-DNA through facilitating strand invasion or by interacting with specific host proteins bound to the DNA (Bako *et al*, 2003; Zhu *et al*, 2003). Collectively, our results suggest that the plant-like T-DNA integration patterns observed in HeLa cells are the result of the combined action of VirD2 and VirE2 proteins.

Outlook/speculation

Our result represents the first example of using a synthetic DNA:protein complex for gene delivery. The virulence proteins improved both the efficiency and the 'quality' of transgene integration. These findings suggest that the synthetic T-DNA complex can function in the context of nuclear import and DNA repair systems of the mammalian cell. Furthermore, they demonstrate that the two bacterial proteins VirD2 and VirE2 are sufficient to result in bona fide T-DNA integration without the requirement for additional bacterial and/or plant-specific factors.

Using a synthetic delivery vector may offer many advantages over using live or replicating agents such as bacteria or viruses. The T-DNA complex does not require containment facilities for its production, as it is incapable of replication and proliferation. Finally, as the virulence proteins were previously shown to facilitate *in vitro* nuclear import of the T-DNA (Ziemiencowicz *et al*, 1999, 2001), the T-DNA complexes may also facilitate gene delivery in somatic cells.

In the course of *Agrobacterium*-mediated transformation, the bacterium delivers the complex into the cytoplasm of the host plant via the type IV secretion apparatus. In our experiments, T-DNA complexes were delivered into HeLa cells using cationic liposomes. Although this delivery method proved to be sufficient, it is probably not optimal for the delivery of protein:DNA complexes. Microinjection, although more technically demanding, may be better suited for the delivery of T-DNA complexes and may be especially useful in applications where a desirable transgene integration pattern must be obtained from just a few recipient cells. One of the inherent problems in generating transgenic animals has been the relatively low transgenesis efficiencies (Hirabayashi *et al*, 2001) compounded by the limited number of oocytes or embryos available. The field of mammalian transgenesis may therefore be the next proving ground for synthetic T-DNA complexes as DNA delivery vehicles.

METHODS

Overexpression and purification of VirE2 and VirD2 proteins. Both proteins were overexpressed in *Escherichia coli* as C-terminal 6 × His fusions and purified using affinity and ion-exchange chromatography (see supplementary information online).

Preparation of ss pre-T-DNA fragments. *DraI/FspI* fragment of plasmid pTK-Hyg (Clontech) carrying a hygromycin resistance cassette was cloned into the *HincII* site of m13-Y3k-s phage (gift from A. Ziemienowicz) downstream of the border. The ssDNA fragment containing the border followed by the hygromycin resistance gene was excised from the phage DNA. Oligonucleotides Y3-*PvuII*3' (TCGCTATTACGCCAGCTGGCGAAAGGGGGA) and Y3-*PvuII*5' (ACCTGTCGTGCCAGCTGCATTAATGAATCG) flanking the desired region were annealed to phage DNA in *PvuII* restriction buffer (10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol). The 2.3-kb pre-T-DNA was excised with *PvuII* and purified on a 0.7% agarose gel.

Synthesis of T-DNA complexes. A 100 ng portion of pre-T-DNA was digested with 100 ng of VirD2 in 20 µl for 30 min at 37 °C in TKM buffer (50 mM Tris-HCl (pH 8.0), 150 mM KCl, 1 mM MgCl₂, 10% glycerol). VirD2-bound T-DNA or naked pre-T-DNA was coated with VirE2 protein. A 100 ng portion of DNA was incubated for 30 min at 4 °C with 2 µg of VirE2 in TKM buffer in a final volume of 21 µl. In gel shift experiments, the minimal amount of VirE2 required to completely coat all ssDNA was determined; a 20:1 VirE2:DNA weight-to-weight ratio was found necessary.

HeLa cell culture and transfections. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) at 37 °C and 7.5% CO₂. A 100 ng portion of ssDNA or T-DNA complexes containing 100 ng of ssDNA was transfected into HeLa cells using Lipofectamine 2000 transfection reagent (Life Technologies). For this, 20 µl of ssDNA or T-DNA processing reaction in TKM buffer was mixed with 500 µl of Opti-MEM medium (Life Technologies). The mixture was added to 20 µl of transfection reagent pre-diluted in 500 µl of Opti-MEM and incubated for 20 min at 20 °C to allow the formation of liposome complexes. The total reaction was added to a 6 cm plate of 70–80% confluent HeLa cells (~2 × 10⁶ cells) grown in 5 ml DMEM with 10% FCS. At 24 h following transfection, the cells were transferred to 15 cm plates and grown in medium containing 100 mg/l hygromycin-B (Sigma) to select stably transfected clones.

Isolation of 5' T-DNA integration junctions by inverse PCR. DNA isolated from hygromycin-resistant lines was digested with *Bam*HI and circularized for 48 h at 16 °C using 2000 U of T4 DNA ligase (NEB). Two pairs of nested primers 5'A, 3'A and 5'B, 3'B (Fig 4) were used in two successive rounds of PCR to amplify the junction fragments (see supplementary information online).

Isolation of 3' T-DNA integration junctions. 3' junctions were recovered by PCR using primer 3'A and an arbitrary, locus-specific primer positioned 0.5–2.0 kb downstream from the integrated T-DNA and designed using sequences positioned downstream of the previously amplified 5' junctions. The PCR products were purified and sequenced directly using the 3'B primer (see supplementary information online).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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