

Genetic transformation of HeLa cells by *Agrobacterium*

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Agrobacterium tumefaciens is a soil phytopathogen that elicits neoplastic growths on the host plant species. In nature, however, *Agrobacterium* also may encounter organisms belonging to other kingdoms such as insects and animals that feed on the infected plants. Can *Agrobacterium*, then, also infect animal cells? Here, we report that *Agrobacterium* attaches to and genetically transforms several types of human cells. In stably transformed HeLa cells, the integration event occurred at the right border of the tumor-inducing plasmid's transferred-DNA (T-DNA), suggesting bona fide T-DNA transfer and lending support to the notion that *Agrobacterium* transforms human cells by a mechanism similar to that which it uses for transformation of plants cells. Collectively, our results suggest that *Agrobacterium* can transport its T-DNA to human cells and integrate it into their genome.

Genetic transformation of plant cells by *Agrobacterium tumefaciens* is the only known natural example of transkingdom DNA transfer. In nature, *Agrobacterium* introduces several oncogenic genes into the host plant, leading to formation of tumors (1), and in the laboratory this microorganism is used widely for plant genetic engineering (2, 3). *Agrobacterium* infection requires the presence of two genetic components located on the bacterial tumor-inducing (Ti) plasmid: the transferred DNA (T-DNA), which is introduced into the plant cell genome, and the virulence (*vir*) region composed of seven loci—*virA*, *virB*, *virC*, *virD*, *virE*, *virG*, and *virH* encoding most components of the protein apparatus for T-DNA transfer. In addition, several bacterial chromosomal virulence (*chv*) genes participate in the early stages of *Agrobacterium* attachment to the plant cells (reviewed in refs. 4 and 5–7).

The infection process begins by chemotactic attraction of *Agrobacterium* toward wounded sites on the host plant, attachment of the bacteria to the plant cell surface, and activation of the T-DNA transfer machinery (5, 6). During the attachment, *Agrobacterium* first loosely binds to the plant surface, and then it produces cellulose fibrils that tighten the binding and allow attachment of additional bacteria (8, 9). To transfer its T-DNA into the host plant cell, the attached *Agrobacterium* uses a complex transport machinery encoded by the *vir* region and specifically activated by signal molecules contained in exudates from wounded-plant cells. The best studied inducer of *vir*-gene expression is acetosyringone (AS), a monocyclic phenolic molecule (10).

Besides its natural plant hosts, *Agrobacterium* has been shown to transfer DNA to other microorganisms such as yeast (11–13), several species of filamentous fungi (14), and a cultivated mushroom (14). However, whether *Agrobacterium* infects higher eukaryotes other than plants remains unknown. Here we address this question by demonstrating that *Agrobacterium* can transform human cells genetically by integrating its T-DNA into the cellular genome. Several aspects of the transformation process characteristic for T-DNA transfer to plants, e.g., bacterial attachment and involvement of virulence proteins (reviewed in ref. 7), also were necessary for T-DNA transfer to human cells.

Materials and Methods

Binary T-DNA Vector. The pNeo plasmid contained a neomycin resistance gene, encoding neomycin phosphotransferase, under

the control of the simian-virus-40 early promoter and Herpes-simplex virus thymidine kinase polyadenylation signal (derived from pEGFP-C1, CLONTECH) subcloned into the Klenow-blunted *Eco*RI and *Hind*III sites of the pPZP221-based binary vector (15), between its right and left nopaline-type T-DNA borders.

Bacterial Strains and Host Cell Lines. *A. tumefaciens*-strain C58C1, harboring Ti-plasmid pGV3850 (16), was used as host for the pNeo binary vector. Strain C58NT1, which does not contain a Ti plasmid (17), was used as control. *Agrobacterium* strains carrying Ti plasmids with individual *vir* loci inactivated by Tn3-HoHo1 transposon insertions were described previously (18). *Agrobacterium* strains ME42 and ME60, with mutated chromosomal virulence genes *chvA* and *chvB*, respectively, were also described (19, 20).

Human HeLa R19 cells, human embryonic kidney (HEK) 293 cells, and clonal pheochromocytoma PC12 neuronal cells, supplied by the Stony Brook Tissue Culture Facility (Stony Brook, NY), were cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air in DMEM (GIBCO/BRL) supplemented with 10% (vol/vol) FBS (GIBCO/BRL). Plant-cell suspension culture of *Petunia hybrida* (line 3704, Hook Vilm, kindly provided by S. Izhar, Volcani Center, Bet-Dagan, Israel; ref. 21) was grown at 25°C and 110-rpm shaking in UM medium (21, 58) under a 12-h daylight cycle. *Petunia* protoplasts were prepared from the cell cultures as described (22).

T-DNA Transfer. A total of 3×10^5 to 5×10^5 human cells was plated on 22×22 -mm coverslips (10^5 cells per coverslip), which were then placed in a 90-mm Petri dish and cultured for 1 day in the presence of 100 µg/ml penicillin/streptomycin mixture (GIBCO/BRL) to avoid contamination. Then, 2 to 5 h before the experiment, the medium was replaced with 10 ml of the fresh medium without antibiotics.

Agrobacterium was grown for 16 h at 28°C and 250-rpm shaking in 5-ml yeast extract/peptone (YEP) medium (23) supplemented with appropriate antibiotics to maintain plasmid vectors (100 µg/ml spectinomycin for pNeo and 100 µg/ml carbenicillin for insertion mutants of Ti plasmid). Then, 100 µl of these bacterial cultures was used to inoculate 5 ml of YEP and was grown for 4 to 6 h at 28°C in the presence of 100 µM of AS [from a 100-mM stock solution in DMSO (Aldrich)] to induce *vir*-gene expression. Note that although minimal medium is more

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Abbreviations: Ti, tumor inducing; T-DNA, transferred DNA; AS, acetosyringone; TAIL, thermal asymmetric interlaced.

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conductive to the induction process than the rich YEP medium, rich media are often used to pregrow *Agrobacterium* during genetic transformation of different plant species (24, 25). Because for HeLa-cell transformation we wished to employ the conditions most close to those used for plant transformation, YEP rather than a minimal medium was used.

For transformation, 100 μ l of preinduced bacterial culture (10^5 – 10^6 bacteria) was added to each tissue-culture plate with human cells, followed by incubation for 48 h at 37°C and 5% CO₂ with 0.1 μ M AS. After coincubation, cells were washed twice with 10 ml PBS and grown in 10 ml DMEM supplemented with 10% FBS, 0.2 mM cefotaxime to kill *Agrobacterium*, and 600 μ g/ml geneticin (G418, GIBCO/BRL) to select for transformants. The selective medium was changed every 2 days; when no bacteria were detected by microscopic examination of the cell cultures, cefotaxime treatment was stopped, and cell cultures were maintained in the presence of geneticin to select for stable transformants. The transformed cell foci were grown to confluence and used for further analyses. Each transformation experiment was performed 2–6 times and usually included 5–10 culture plates per system. Microsoft EXCEL software was used to calculate standard deviations between the results of individual experiments.

Bacterial Attachment. HeLa cells (10^5) were cultured for 16 h on a 22 \times 22-mm coverslip in a 6-well dish and transferred to the fresh medium. Then, 2 h later, bacterial cultures were added and coincubated with HeLa cells for 16 h at 37°C as described above for the T-DNA transfer experiments. For attachment to petunia protoplasts, the coincubation was performed at 28°C in suspension cultures. After incubation, the samples were examined and recorded by using a Zeiss Axioskop microscope equipped with MTI CCD-300T-RC video camera (for HeLa cells) or Axioplan 2 microscope equipped with Nikon Culitix 950 camera (for petunia protoplasts).

Southern Blot Analysis of T-DNA Transgenes in HeLa Cells. Genomic DNA was extracted from six 90-mm plates of confluent cell cultures derived from a single stably transformed cell line by using the High Pure PCR Template Preparation kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. The purified DNA (20 μ g) was digested with *Eco*0109I, *Hind*III, *Eco*RI, *Bam*HI, *Pst*I, or *Sal*I restriction enzymes. In the T-DNA region of pNeo binary vector, which is expected to be transferred and integrated into the HeLa-cell genome, there are two cleavage sites for *Eco*0109I, a single cleavage site for *Hind*III, *Eco*RI, *Bam*HI, and *Pst*I, and no sites for *Sal*I. The digested DNA was analyzed by Southern blot hybridization by using standard techniques (26) and a 740-bp radiolabeled probe corresponding to the neomycin resistance gene of pNeo; this probe fragment did not contain the recognition sites for restriction enzymes used to digest the genomic DNA.

Thermal Asymmetric Interlaced (TAIL) PCR Amplification of the Integration Junction. TAIL PCR was performed as described for mapping of genomic sequences flanking T-DNA insertions in *Arabidopsis* (27). Briefly, genomic DNA from the same stably transformed HeLa cell line used for the Southern blot analysis was purified (see above), and the putative T-DNA integration junction was amplified by using three consecutive rounds of PCR. The first PCR was carried out in a 50- μ l volume containing 20 ng DNA, 0.1 nM dNTP, 2.5 mM of the T-DNA-specific sense primer TR1 (5'-GCACTGGCCGTCGTTTACAACG-3'), 2.5 mM of a degenerate antisense primer AD2 (5'-NTCGASTWTS-GWGTT-3', in which N is A or C or G or T, S is C or G, and W is A or T), and 2 units of TKARA EX-*Taq* polymerase (Panvera, Madison WI) with 5 μ l of EX-*Taq*10X reaction buffer.

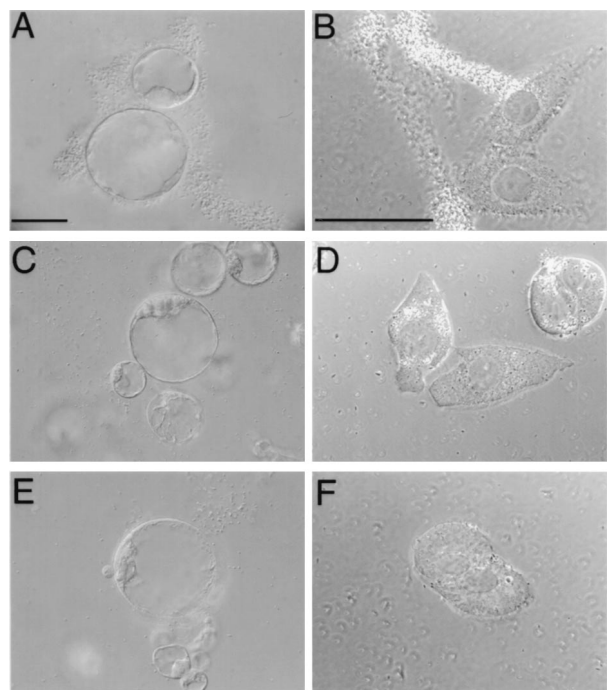


Fig. 1. *Agrobacterium* attachment to petunia protoplasts and HeLa cells. (A and B) Wild-type *Agrobacterium* incubated with petunia protoplasts and HeLa cells, respectively. (C and D) *chvA* mutant of *Agrobacterium* incubated with petunia protoplasts and HeLa cells, respectively. (E and F) *chvB* mutant of *Agrobacterium* incubated with petunia protoplasts and HeLa cells, respectively. (Bars = 50 μ m.)

After 5 min of denaturation at 92°C, the junction fragment was amplified for 5 cycles of 60 sec at 94°C, 60 sec at 62°C, and 2.5 min at 72°C followed by 22 cycles of 30 sec at 94°C, 60 sec at 68°C, 2.5 min at 72°C, 30 sec at 94°C, 60 sec at 44°C, and 2.5 min at 72°C. The resulting mixture was diluted 10 times and subjected to the second PCR under the same conditions but by using a downstream-nested T-DNA-specific sense primer TR2 (5'-GGGAAAACCCTGGCGTTACCC-3'). Then 10 times-diluted products of the second reaction were amplified the third time by using another downstream-nested T-DNA-specific sense primer TR3 (5'-CCTTTCGCCAGCTGGCGTAATAGCG-3'). The resulting amplified T-DNA–HeLa-DNA junction fragment was subcloned into a pBluescript SK(+) (Stratagene) plasmid, and its DNA sequence was determined.

Results

***Agrobacterium* Attachment to HeLa Cells.** Infection of host cells by *Agrobacterium* must begin from attachment of the bacteria to the host cell. Thus, we examined the ability of *Agrobacterium* to associate with HeLa cells and compared it to the well characterized *Agrobacterium* plant-cell binding (9, 28, 29). Fig. 1A shows that *Agrobacterium* attaches to petunia protoplasts, enmeshing itself and the plant cells into aggregates (9, 28, 29). Fig. 1B demonstrates a similar attachment pattern with characteristic bacterial aggregates at and around the host cells observed after incubation of *Agrobacterium* with HeLa cells.

To investigate whether *Agrobacterium* binding to HeLa cells requires the same bacterial factors as its attachment to plant cells, we used two avirulent *Agrobacterium* mutants, *chvA* and *chvB*. These mutations, which are located on the bacterial chromosome, result in inability of the bacteria to bind to host cells (19, 20). Neither *chvA* nor *chvB* mutants (Fig. 1C and E, respectively) attached to plant protoplasts or HeLa cells (Fig. 1D and F, respectively).

Table 1. Genetic transformation of human cells by *Agrobacterium*

Plasmids	Host-cell type	Total number of geneticin-resistant transformants	Efficiency of transformation per 10 ⁶ cells, \pm SD*
pTi-pNeo in <i>Agrobacterium</i>	HeLa	140	17 \pm 4
pTi-pNeo in <i>Agrobacterium</i> without preinduction	HeLa	74	9 \pm 2
pNeo in <i>Agrobacterium</i>	HeLa	0	<0.1
pNeo alone	HeLa	0	<0.1
pNeo Ca-Phosphate	HeLa	179	19 \pm 6
pTi-pNeo in <i>Agrobacterium</i>	HEK393 (kidney)	167	18 \pm 6
pNeo in <i>Agrobacterium</i>	HEK393 (kidney)	0	<0.1
pNeo alone	HEK393 (kidney)	0	<0.1
pTi-pNeo in <i>Agrobacterium</i>	PC12 (neuronal)	112	12 \pm 3
pNeo in <i>Agrobacterium</i>	PC12 (neuronal)	0	<0.1
pNeo alone	PC12 (neuronal)	0	<0.1

*Microsoft EXCEL software was used to calculate standard deviations between two to six individual experiments, each of which included 5–10 culture plates per system.

T-DNA Transfer from *Agrobacterium* to Human Cells. To establish whether *Agrobacterium* transfers T-DNA to human cells, we constructed the binary vector pNeo. This plasmid carries a T-DNA that contains a neomycin resistance gene driven by the Simian-virus-40 early promoter, which is specific for mammalian cells. pNeo was introduced then into the *Agrobacterium* helper-strain C58C1, harboring the Ti plasmid pGV3850 (16), which serves as a source of *vir*-gene products in a binary transformation system. For control experiments, pNeo was introduced into the *Agrobacterium* C58NT1 strain cured of its Ti plasmid (17).

Coincubation with AS-induced *Agrobacterium* resulted in genetic transformation of HeLa cells as evidenced by formation of geneticin-resistant cell lines (Table 1). The transformation frequency was comparable to that of calcium phosphate stable transfection of mammalian cells (Table 1) but lower than the highly efficient lipofectin-mediated transformation method (30). The resistance was retained through many cell duplications (usually the resistant lines were maintained for 2 to 6 weeks and then discarded), suggesting stable integration of the transgene. Incubation with *Agrobacterium* lacking the Ti plasmid but harboring pNeo never gave rise to antibiotic-resistant HeLa cells (Table 1), indicating that presence of the *vir* genes was essential for genetic transformation. The requirement for the Ti plasmid also indicates that potentially spontaneous uptake by HeLa cells of pNeo DNA released from bacteria was not responsible for transformation. Also, the expression of neomycin resistance could not be attributed to contaminating bacteria, because this gene in the pNeo vector is driven by the simian-virus-40 early promoter; such viral promoters are specific strictly for animal cells (31) and are not expressed in bacteria. As expected, no transformants were obtained when the cells were incubated with the purified pNeo plasmid alone, in the absence of *Agrobacterium* (Table 1). Surprisingly, however, *Agrobacterium* stably transformed HeLa cells, albeit with lower efficiency, even in the absence of exogenously added *vir*-inducer AS (Table 1), suggesting that human cells and/or their growth medium may have a certain capacity for *vir*-gene induction. Alternatively, two different mechanisms may be responsible for transformation of HeLa cells by *Agrobacterium*: *vir* dependent and *vir* independent.

Next, we examined the ability of *Agrobacterium* to infect other human cell lines. Table 1 shows that HEK393 cells and neuronal PC12 cells also were susceptible to genetic transformation by *Agrobacterium*, forming geneticin-resistant transformants. Similarly to HeLa cells, transformation of HEK393 and PC12 depended on the presence of the Ti plasmid (Table 1).

Evidence for T-DNA Integration into the HeLa-Cell Genome. Genomic DNA was isolated from one of the stable geneticin-resistant

HeLa cell lines (transformant 7–14), digested with *SalI*, *PstI*, *BamHI*, *EcoRI*, *HindIII*, or *Eco0109I* restriction enzymes, and analyzed by Southern blot hybridization by using the neomycin resistance gene as probe (Fig. 2A). For control experiments, DNA isolated from a wild-type untransformed HeLa-cell line was used. Fig. 2 shows that although no signal was detected in the DNA from wild-type cells (B), a neomycin-specific signal was present in the transformed cell genome (C). Digestion with *SalI*, which has no recognition sites within the entire T-DNA region of pNeo, produced a single high-molecular-weight band (Fig. 2B, lane 1), indicating a single integration site for the T-DNA.

To estimate the number of T-DNA inserts within this integration site, we performed digestion with *PstI*, *BamHI*, *EcoRI*, and *HindIII*, each of which has a single recognition site in the T-DNA but outside of the neomycin resistance gene. The resulting single bands with variable sizes (Fig. 2B, lanes 2–5, respectively) suggested integration of a single T-DNA copy. Also, that the size of these fragments differed from that predicted for restriction digestion of the complete pNeo plasmid (14,727 bp) or its free unintegrated T-DNA region (8,727 bp; Fig. 2A), suggests that these fragments represent the neomycin resistance gene linked to the chromosomal sequences. In the undigested samples, the signal coincided with very high-molecular-weight DNA (data not shown), confirming integration of the T-DNA into the chromosomal DNA. In control digestion with *Eco0109I*, which has two recognition sites bracketing the neomycin resistance gene, a fragment corresponding to the predicted T-DNA fragment (Fig. 2A) was released (Fig. 2B, lane 6).

To confirm the integration event further, we used TAIL PCR (ref. 27; outlined in Fig. 3A) to isolate the T-DNA-HeLa DNA junction from the same cell line with a single T-DNA insertion analyzed in Fig. 2. Unlike integration of transposable elements, which is generally precise, T-DNA insertions are imprecise; nevertheless, in plants, the junction points at the right T-DNA border are more consistent and occur close to or within the border sequence, whereas the left border junctions can be spread over much longer segments, often deleting the border itself (reviewed in refs. 32–34). Similar preservation of the right border region relative to the left border region was observed during *Agrobacterium*-mediated transformation of yeast (13) and filamentous fungi (14). Thus, we focused on isolation of the right border junction site. Our TAIL PCR experiments using DNA from wild-type untransformed HeLa cells as negative control produced no specific fragments (data not shown), whereas the DNA from the transformed HeLa-cell line yielded only one

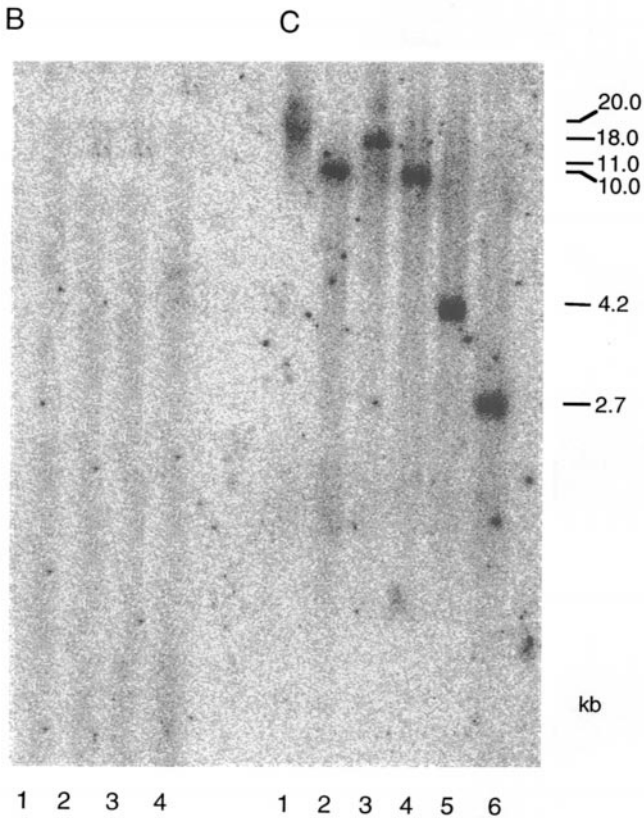
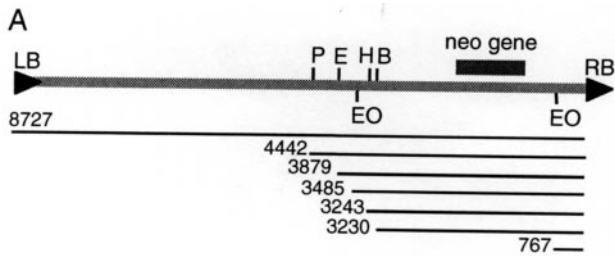


Fig. 2. Southern blot analysis of a geneticin-resistant HeLa cell line stably transformed by *Agrobacterium*. (A) Organization of the pNeo plasmid. The T-DNA region of pNeo with the restriction sites used for the Southern blot analysis is shown. P, H, E, B, and EO indicate *Pst*I, *Bam*HI, *Eco*RI, *Hind*III, or *Eco*0109I restriction enzyme sites, respectively. LB and RB indicate left and right T-DNA borders, respectively. Thin lines indicate the length of the entire T-DNA and distances of each restriction site from RB in base pairs. The location of the neomycin resistance (neo) gene is shown. The pNeo backbone (6 kb, not shown) outside of the T-DNA region is that of the parental pPZP221 vector (ref. 15; GenBank accession no. U10490). (B) Wild-type untransformed cell line. (C) Stably transformed cell line. Lanes 1 to 6 show digestions with *Sal*I, *Pst*I, *Bam*HI, *Eco*RI, *Hind*III, or *Eco*0109I restriction enzymes, respectively. The blots were hybridized with a 740-bp radiolabeled probe corresponding to the neomycin resistance gene of pNeo (see A). The sizes of the restriction-fragment bands were calculated based on standards and are indicated on right in kilobase pairs (kb).

fragment that contained both HeLa and *Agrobacterium* sequences (Fig. 3A).

The DNA sequence of this fragment was determined and compared with the sequence of the right T-DNA-border region of the pNeo binary vector and to the known chromosomal DNA sequences of the human genome (Fig. 3B). This analysis identified a pNeo sequence within the junction fragment (open boxes in Fig. 3B). It also demonstrated that, from the 25-bp right border sequence of pNeo (black box in Fig. 3B), four bases remained at the junction site, consistent with the observations

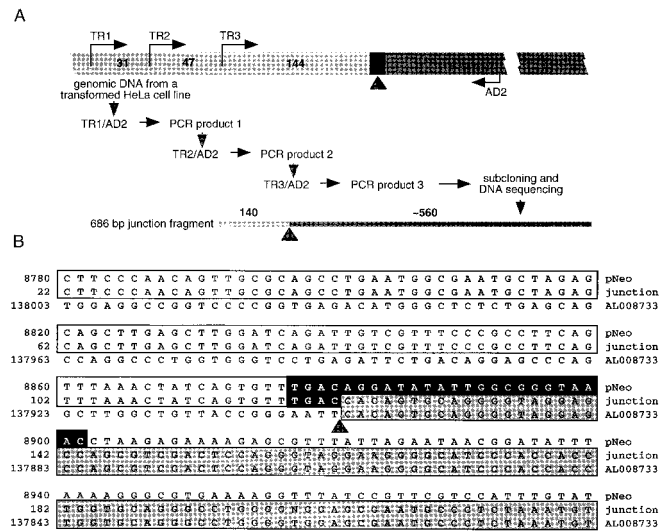


Fig. 3. Cloning of the T-DNA-HeLa DNA integration junction. (A) TAIL-PCR cloning strategy. Wide bar illustrates the genomic DNA from a transformed HeLa cell line used as a template, black rectangle indicates the region of the right T-DNA border, and numbers indicate distances between the nested sense PCR primers TR1 and TR2, TR2 and TR3, and TR3 and the right border. AD2 is the degenerate antisense primer expected to anneal within HeLa-cell DNA. Narrow bar indicates the amplified junction fragment in outline, and numbers indicate the size of its corresponding T-DNA and HeLa-DNA components. Light and dark segments of both bars indicate T-DNA and HeLa-cell DNA, respectively, whereas the arrowhead indicates the integration point between the T-DNA and HeLa-DNA. (B) Nucleotide sequence alignment of the right T-DNA-border region of pNeo, the isolated integration junction from an *Agrobacterium*-transformed HeLa-cell DNA, and the human genomic DNA (GenBank accession no. AL008733). All sequences are shown in the 5' to 3' direction. The pNeo sequence is based on the right border region of the parental pPZP221 vector (15) (GenBank accession no. U10490) and the human DNA sequence is from clone RP1-163G9 from chromosome 1p36.2-36.3. The consensus nopaline-type right T-DNA-border sequence, described in refs. 33 and 57, is indicated by a black box. Homology of the junction fragment to pNeo is indicated by open boxes and to the human DNA by shaded boxes. Arrowhead indicates the integration point at which the right T-DNA border is fused to the human DNA. Note also that the DNA sequences upstream of the position 22 and downstream of the position 221 in the 686-bp junction fragment were identical to the corresponding regions of the pNeo and AL008733 DNA, respectively (data not shown).

that the nopaline-type right border is cleaved between its third and fourth base during generation of the transferable copy of the T-DNA, the T strand (35) (potentially, the fourth border base of the junction may have been derived from a duplication of the adjacent identical base in the HeLa DNA; see Fig. 3B).

That the cleaved right border sequence was found at the integration junction and, importantly, that no pNeo sequences to the right of the right T-DNA border, i.e., non-T-DNA sequences, were found in the junction site (Fig. 3B) is indicative of the true T-DNA integration rather than nonspecific insertion of plasmid DNA. Fig. 3 also shows that, at the junction point (arrowhead), the T-DNA sequence was linked to a HeLa-DNA sequence virtually identical to the region between bases 137,901 and 137,583 of the human DNA sequence from clone RP1-163G9 on chromosome 1p36.2-36.3 (GenBank accession no. AL008733; shadowed boxes), directly demonstrating integration of the right T-DNA border into the host cell genomic DNA. There was no significant homology between the inserted pNeo sequence and its integration site within the HeLa-cell DNA (Fig. 3B), suggesting that T-DNA integration in mammalian cells, as in fungi (13, 14) and plants (34), is sequence-independent.

We could not compare the TAIL PCR data directly to the Southern analysis data, because the length of the amplified junction fragment (686 bp) was significantly smaller than the smallest restriction fragment detected in Southern blotting (≈ 2.7 kb, see Fig. 2, lane 6). Nevertheless, the nucleotide sequence of the junction fragment (Fig. 3B) was consistent with the Southern blot data, because it did not contain *Eco*0109I, *Hind*III, *Eco*R1, *Bam*HI, *Pst*I, or *Sal*I restriction sites, which if present would have produced a different hybridization pattern on the Southern blot. Collectively, our Southern blot results and sequence analysis of the integration junction suggest that *Agrobacterium* can transport its T-DNA to HeLa cells and integrate it into their genome.

Effects of *vir*-Gene Mutations on T-DNA Transfer to HeLa Cells. Most protein products of the *vir* genes play critical roles in the infection process and thus are indispensable for transformation of plant cells by *Agrobacterium*. If *Agrobacterium* transforms plant and human cells by the same molecular mechanism, HeLa-cell transformation should be blocked by mutations inactivating the major *vir* genes. Our observations that an *Agrobacterium* strain lacking its Ti plasmid, which supplies the *vir* genes that encode the T-DNA transfer machinery, was unable to transform all three human cell lines (Table 1) support this notion. We directly confirmed the involvement of *vir* genes in transformation of HeLa cells by using a series of *Agrobacterium* mutant strains, in which one of the five major *vir* loci, *virA*, *virB*, *virG*, *virD*, or *virE*, was inactivated by a Tn3-HoHo1 transposon insertion (18). Specifically, *virA* and *virG* were inactivated in pTi237 and pTi19 mutants, respectively, whereas *virB*, *virD*, and *virE* were inactivated in two independent mutants each, i.e., pTi243 and pTi30, pTi311 and pTi304, and pTi361 and pTi358, respectively (18, 36). In addition, *Agrobacterium* mutants ME42 and ME60 in the chromosomal virulence genes *chvA* and *chvB*, respectively (19, 20), also were tested.

These experiments demonstrated that all tested strains with mutations in the *vir* or *chv* genes lost their transforming ability, producing no geneticin-resistant cell lines under our experimental conditions. In control experiments, wild-type *Agrobacterium* infected HeLa cells, generating 62 geneticin-resistant transformants, corresponding to the transformation efficiency of 16 ± 3 stable transformants per 10^6 cells. Thus, *Agrobacterium*-mediated transformation of human cells may involve at least some of the specific virulence protein components of the T-DNA-transport machinery that are required for transformation of plant cells.

Discussion

Agrobacterium cells attached to both HeLa cells and plant protoplasts. Furthermore, *Agrobacterium* binding to, and genetic transformation of, HeLa cells depended on the presence of *chvA* and *chvB* genes, known to be required for bacterial attachment to plant hosts (19, 20). Generally, pathogenic bacteria have been proposed to employ similar mechanisms to attach themselves to the surfaces of plant and animal host cells (37). For example, plant vitronectin-like proteins likely function as host cell receptors used by *Agrobacterium* (37), and mammalian vitronectins also play a role in host colonization by several pathogenic bacteria, such as streptococci, *Staphylococcus aureus*, etc. (38–40). Thus, although binding in itself does not imply specific host-pathogen recognition, it is consistent with the ability of *Agrobacterium* to stably transform human cells. Such genetic transformation was demonstrated directly by production of transgenic mammalian cell lines resistant to neomycin after coinoculation with *Agrobacterium*.

Sequence analysis of the pNeo–HeLa DNA junction showed that the integration event occurred at the right T-DNA border, suggesting bona fide T-DNA transfer and lending support to the notion that *Agrobacterium* transformed HeLa cells by a mech-

anism similar to what it uses for transformation of plants cells. Indeed, *Agrobacterium*-mediated HeLa-cell transformation required the activity of *vir* genes necessary for plant transformation (18). Specifically, mutants in the *virA*, *virB*, *virG*, *virD*, and *virE* genes, which are avirulent on plant hosts (18), also failed to transform HeLa cells. During *Agrobacterium* infection of plant cells, these *vir* genes play critical roles. Specifically, VirA and VirG sense plant phenolics and promote transcriptional activation of the *vir* genes (reviewed in ref. 41), VirB proteins are required for T-DNA export from *Agrobacterium* into the host cell (reviewed in ref. 42), and VirD and VirE proteins generate and associate with a mobile single-stranded copy of the T-DNA and mediate its import into the host cell nucleus and possibly its integration into the host genome (reviewed in ref. 7). Thus, *Agrobacterium* likely uses its plant-transforming protein machinery for transformation of HeLa cells. This assumption implies that such basic processes as nuclear import and DNA integration are similar enough between plant and animal cells to be taken advantage of by *Agrobacterium*. Indeed, the VirD2 protein localizes to the cell nucleus both in plant (43–47) and animal systems (48, 49), and its cellular receptor, AtKAP α , also is conserved between these organisms (50). Also, although its nuclear import in plant and animals remains controversial (48, 49), VirE2 together with VirD2 facilitate nuclear import of DNA into mammalian cell nuclei *in vitro* (48). For example, in mammalian cells, VirE2 may act to shape the T-DNA into a transferable form and/or protect it from cellular nucleases (48, 51, 52).

An interesting aspect of *Agrobacterium*-mediated transformation of mammalian cells is that it occurred at 37°C. Previously, exposure to high temperatures has been shown to reversibly inhibit VirA, which is involved in perceiving the *vir*-inducing plant signals (53), and/or other components of the T-DNA transfer machinery (54). Our transformation protocol includes pregrowth of *Agrobacterium* at 28°C in the presence of the *vir*-inducer AS. Because at 28°C *vir* genes are well expressed (18), the preinduced *Agrobacterium* cells may be competent already for transformation, allowing the T-DNA transfer even at higher temperatures.

Our results also suggest that uninduced *Agrobacterium* can still transform HeLa cells, although with significantly lower efficiency. This transforming activity may be due to various reasons such as *vir*-gene induction by the components of the HeLa-cell growth medium, e.g., phenol red pH indicator or FBS, or even HeLa-cell exudates. Furthermore, a secondary *vir*-gene-independent mechanism such as taking up of *Agrobacterium* by endocytosis may contribute also to the transformation process. However, because the T-DNA integration occurred at the predicted specifically nicked border sequence, the VirD2 and VirD1 proteins, which potentiate this nick (reviewed in ref. 7), most likely were involved. This notion is strengthened further by our observations that *vir*-gene mutants of *Agrobacterium* or *Agrobacterium* carrying the T-DNA-containing pNeo plasmid but not the *vir*-genes-containing Ti plasmid were unable to transform HeLa cells. Clearly, additional experiments will be required to elucidate the exact mechanism by which *Agrobacterium* transforms mammalian cells. Presently, however, there is little doubt that *Agrobacterium* T-DNA can be transferred to and integrated into the mammalian cell genome.

That *Agrobacterium* carrying a neomycin resistance gene within its T-DNA generated stable antibiotic-resistant lines of human cells expands its potential host range from plants, yeast, and filamentous fungi to mammalian cells. Here, transformation of human cells has been observed in laboratory conditions; whether it may be relevant biologically in nature remains unknown. Interestingly, *Agrobacterium* or *Agrobacterium*-related species have been suggested to be involved in several human diseases (55, 56).

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