# Improvements in adenovirus technology : aiming at replication specificity and vector integration

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# CHAPTER 6

**Evaluation of an *A. tum.*-derived system for integration of adenovirally delivered transgene cassettes in mammalian cells**

# Evaluation of an *Agrobacterium tumefaciens*-derived system for integration of adenovirally delivered transgene cassettes in mammalian cells

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**Recombinant Ads are widely used to deliver foreign genes to a wide range of host cells. A major limitation is the relative short duration of gene expression, especially in dividing cells. Genomic integration of the transgene should increase the duration of transgene expression and the efficacy of Ad-mediated gene therapy. *Agrobacterium tumefaciens* is a soil bacterium that can transfer T-DNA into dicotyledonous plant. Moreover it has been shown to be capable of successfully transferring and integrating T-DNA into yeast, fungal and mammalian cells. In plants, the bacterial proteins VirD1 and VirD2 are sufficient for integration of a selection cassette flanked by the left and right borders. The relative simplicity of the integration system may provide a means to create integrating adenoviral vectors. We have studied the possibilities of VirD1 and VirD2-mediated integration of adenovirally delivered transgenes. Co-delivery of VirD1 and VirD2 resulted in a 60-fold increase in the integration efficiency. We demonstrate that the transgenes are stably integrated into the chromosomal DNA.**

Human adenoviruses (HAdV) are double-stranded DNA viruses that are frequently used as gene-delivery vectors in biomedical research and in experimental gene therapy. Although efficient, adenovirus vectors usually yield transient gene expression. This is, in part, due to loss of the transgene upon cell division. For this reason HAdVs are less suited for applications in which persistent transgene expression is required.

This problem has provoked the use of other gene-delivery vectors that have the capacity to stably integrate the transgene into the host cell genome. The oncoretrovirus, lentivirus and adeno-associated virus-derived vectors are successfully used as gene-transfer vectors. However, these viruses impose stringent limitations on the size of the transgene. Here we describe the development and evaluation of an *Agrobacterium tumefaciens* (*A.tum.*)-derived system that can be used for adenovirus- mediated integration of the transgene cassette.

*Agrobacterium tumefaciens* is a soil bacterium that can cause 'crown gall' tumors in dicotyledonous plants. The formation of 'crown galls' is the result of the transfer of single-stranded DNA molecules (T-DNA) from the bacteria into the nucleus of plant cells [1-3] where it is randomly integrated into to the plant cell genome (Agrolystic integration). Expression of genes located on the T-DNA lead to the production of plant hormones that induce cell division, resulting

in the formation of 'crown gall' tumors. In addition to plant, *A.tum.* mediated T-DNA transfer has been shown in fungal [4-7], yeast [8-10], and HeLa cells [11].

The T-DNA consists of a region on the tumor inducting plasmid (Ti-plasmid), which is flanked by a 24-bp left border (LB) and right border (RB) repeat. The virulence proteins D1 and D2 (VirD1 and VirD2) bind these borders, and VirD2 nicks a single strand in the recognition sequence. Subsequently, the 5' terminus of the T-DNA is covalent bound to $Tyr^{29}$ of VirD2 [12,13] after which the T-DNA is displaced from the Ti-plasmid. The T-DNA translocates to the plant cytosol via pores between *A.tum.* and the plant cell membrane formed by the VirB proteins. In the plant cytosol the T-DNA is coated with VirE2 [14] and this complex translocates to the nucleus, a process that is dependent on the presence of both the VirD2 and VirE2 proteins [15,16]. The integration of the T-DNA in plant cells takes place via illegitimate recombination facilitated by plant enzymes [17]. Most likely, VirD2 and VirE2 do not play an essential role in the actual integration process.

Detailed T-DNA integration studies in yeast resulted in the finding of a major integration pathway dependending on non-homologous end-joining proteins (Yku70, Rad50, Mre11, Xrs2, Lig 4 and Sir4) and a minor inter-telomeric integration pathway that requires Rad50, Mre11 and Xrs2. Cells lacking Yku70 were not susceptible for T-DNA integration indicating that this protein plays an essential role in this process. It remains to be established if these proteins also play an essential role in human cells. While in plant, agrolystic integration is predominantly at random, in yeast homologous recombination is the prevalent integration method. Recently, a ten to hundred-fold increase in targeted integration was reported for Arabidopsis thaliana expressing the yeast Rad54 protein, a SWI/SNF chromatin remodeling factor, suggesting an role for chromatin remodeling as a rate-limiting step in homologous recombination in plant [18].

The *A.tum*. T-DNA transfer and integration machinery requires a complex interplay of multiple proteins, which are involved in regulation, synthesis and transport of the T-DNA. When delivered by Ad vectors, only proteins involved in the generation of T-DNA are required since the Ad genome is already efficiently transported to the host nucleus. The minimal requirements for *A.tum.* integration has been studied by Hansen and colleges [19]. In maize cells, co-delivery of plasmids encoding VirD1, VirD2 and a LB/RB flanked neomycin phosphotransferase II (nptII) expression cassette resulted in integration of the T-DNA and formation of Neomycin resistant ($Neo^R$) colonies. Thus VirD1 and VirD2 seem to be sufficient for integration of T-DNA. However, when T-DNA complexes were injected in the cytosol of human cells, VirE2 was also required for efficient translocation of the T-DNA complex to the nucleus [20]. Nuclear localization of T-DNA is most likely dependent on VirD2 since a VirD2 mutant lacking the nuclear localization signal (NLS) was defective in nuclear translocation in the presence of VirE2[16]. VirE2 might play an important role in the protection of T-DNA against degradation. Although VirE2 is not required for integration, it doubled the integration efficiency in maize when cells were transected with VirD1, VirD2, VirE2 and the T-DNA encoding plasmid [19].

Here we describe the development and characterization of an *A.tum.*-based system for the chromosomal insertion of transgenes delivered with HAdVs-vectors.

## Materials and methods

**Plasmids.** The genes encoding VirD1 and VirD2 were isolated by PCR and cloned in pCDNA3.1+ (pCD-VirD1 and pCD-VirD2 respectively) and pSuperCatch (pSC-Flag-VirD1 and pSC-Flag-VirD2 respectively) which results in a fusion construct containing an amino-terminal Flag epitope. The VirD1 and Flag-VirD2 genes were cloned into pShuttle-CMV which results after homologous recombination with pAdEasy-1 in Ad.Flag-VirD1 and Ad.VirD2 respectively. Ad.RB-Neo

was generated by constructing a pShuttle based vector containing the Neomycine resistance cassette and E.coli origin of replication from plasmid pRSV-Neo, as well as the RB from plasmid pSDM14. The pShuttle-based vectors were recombined with pAdEasy-1 to generate infectious genomes.

**Virus production and isolation.** Recombinant Ads were generated by electroporating E.coli BJ5183 cells with pAdEasyI and PmeI digested pShuttle based vectors and selected for homologous recombination as described [21,22]. The resulting rAdV-5 plasmids were PacI-digested and transfected into 911 or 911-H2pTP cell lines to generate virus. Approximately 14 days posttransfection, cells were isolated in phosphate-buffered saline (PBS), 2% Horse Serum (HS), and freeze/thawed three times to release the virus. The cell supernatant was used to infect near-confluent PER.C6 cells. PER.C6 cells were infected with Ads in DMEM supplemented with 2% HS, antibiotics, and 3 g/l glucose, in a 5% CO2 atmosphere at 37°C. Two hours post infection the inoculum was replaced with DMEM supplemented with 8% FCS, antibiotics, and 3 g/l glucose, and cultured in a 5% CO2 atmosphere at 37°C. Approximately 48 hours post infection, cells were harvested in PBS supplemented with 2% HS and freeze/thawed to release virus particles. Virus was isolated from the supernatant by CsCl ultracentrifugation, dialyzed against Sucrose buffer and stored at -80 °C.

**Cell culture and colony staining.** Human Osteosarcoma cells (U2OS), Human Caucasian larynx carcinoma cells (Hep2), Ad5-E1-transformed human embryonic retina cell lines 911 and PER.C6 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 8% FCS, antibiotics, and 3 g/l glucose in a 5% CO2 atmosphere at 37°C. To determine integration efficiencies, G418-resistant colonies were fixed with methanol/acidic acid (3:1) and stained with 3% Giemsa in 1 mM $Na_2HPO_4$ pH 7.4.

**Immunofluorescence.** Proteins containing a N-terminal Flag epitope fusion were detected by immunefluorescence using 1:400 diluted monoclonal mouse a Flag antibody (muFlag M2; Kodak, New Haven, CT) as primary antibody and Fluoresceinisothiocyanate-labeled goat anti-mouse as secondary antibody (GαM-Fitc; Jackson Immunoresearch Laboratories, WestGrove, PA). Cell nuclei were detected by staining genomic DNA with 1 µg/ml 2,4-diamino-2-phenylindole (DAPI), 2% 1,4 diazabicyclo[2,2,2]-octane and 0.1 M Tris–HCl pH 8.0 in glycerol.

**Southern analysis.** For the detection of genomic integrations, 10 µg of genomic DNA from monoclonal cell lines was isolated, digested with HindIII and size fractionated on a 1% agarose gel. The integrated vector DNA was detected by Southern blotting with a radioactive labeled fragment of the nptII gene as a probe.

## Results

The bacterium *A.tum.* utilizes an efficient system to transfer ssDNA tumor-inducing DNA (T-DNA) to the nucleus of eukaryotic cells where the T-DNA is stably integrated. The *A.tum*.-derived VirD1 and VirD2 proteins are directly involved in nicking border sequences in the double-stranded DNA. This is the first step in T-DNA synthesis. If the Ti plasmid is present in the cell, the VirD1 and VirD2 proteins are sufficient to generate T-DNA that can integrate in the host cell genome [19,23]. If a transgene cassette flanked by the border sequences is present in the nucleus of mammalian cells, VirD1 and VirD2 could generate T-DNA, which may be sufficient to facilitate integration of the transgene cassette.

To study whether VirD1 and VirD2 are sufficient for T-DNA formation and integration in mammalian cells, we constructed expression vectors for wt and flag-tagged VirD1 and VirD2 (i.e. pCD-*VirD1*, pCD-*VirD2*, pSC-*Flag-D1* and pSC-*Flag-D2*). In addition, two adenoviruses were generated, Ad.*Flag-VirD1* and Ad.*VirD2* (Figure 1). In *A.tum.* the VirD1 protein is not required for the translocation of the T-DNA to eukaryotic nuclei and therefore VirD1, in contrast to VirD2, does not contain a nuclear localization signal. In our adenoviral approach however, the viral DNA is delivered into the nucleus thus requiring both proteins to be present in the nucleus. To evaluate the localization of VirD1, the localization of the VirD1 and VirD2 proteins were tested in U2OS cells. The Flag-tagged Vir proteins were detected by immune-fluorescence confocal laser-scan microscopy using anti-Flag antibodies (Figure 2). Transfection of the Flag-VirD1 construct resulted in predominant cytoplasmic aggregates of VirD1 with little of the protein located in the nucleus (Figure 2A). Infection of U2OS cells with Ad.Flag-D1 resulted in varying expression levels (Figure 2B). Most

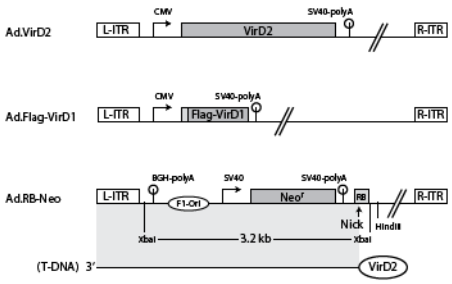


***Figure 1.*** *Schematic presentation of the recombinant Adenoviral constructs. Both Ad.Flag-VirD1 and Ad.VirD2 contain a Cytomegalovirus Immediate Early promoter (CMV) driven expression cassette and the SV40 poly-adenylation signal. The VirD1 gene is fused with a Flag-epitope at the N-terminus. The Ad.RB-Neo contains the Neomycin phosphotransferase II gene flanked by the SV40 promoter and SV40-poly adenylation signals. Downstream of the expression cassette is the A. tum. Right Border (RB) located. The VirD2 induced nick site is located and the bottom strand so the T-DNA synthesis takes place in the direction of the Ad left ITR. The synthesized T-DNA is depicted below. The HindIII restriction site used in the Southern analysis is indicated.*

cells had low amounts of Flag-D1. In these cells, the protein was localized in the nucleus while in higher expressing cells FlagVirD1 was both located in the nucleus and the cytosol (Figure 2A).

When Flag-VirD1 was co-expressed with VirD2, the Flag-VirD1 protein levels appeared higher than with Flag-VirD1 alone. However, in addition to the cytoplasmic aggregates a more diffuse staining pattern was observed in the nucleus when VirD2 is present. Co-infection of U2OS cells with both Ad.Flag-VirD1 and Ad.VirD2 resulted in similar results (Figure 2B). Flag-VirD1 protein levels seemed higher in VirD2 co-infected cells and more cells expressing low levels of nuclear Flag-VirD1 were detected. The VirD2 protein contains a bipartite nuclear localization signal and as expected, Flag-VirD2 proteins were almost exclusively found in the nucleus. Cotransfections with VirD1 did not alter Flag-VirD2 localization significantly. The nuclear localization of Flag-VirD1 after adenoviral delivery made us decide to use this method to test the integration efficiency.

To test the Flag-VirD1 and VirD2-mediated integration, we constructed the adenovirus vector Ad.RB-*Neo* (see Figure 1). The Ad.RB-*Neo* construct contains the Neomycine Phosphotransferase II gene as a resistance marker flanked by the left ITR and the Right Border (RB). The VirD1/VirD2 complex should induce a nick at the RB sequence, resulting in the synthesis of a T-DNA in the direction of the adenovirus left ITR. Successful integrations were determined by counting G418 resistant ($Neo^R$) colonies in 6-well dishes containing U2OS cells. To determine the level of background integration of the Ad.RB-*Neo* genome, cells were infected with Ad.RB-*Neo* (moi 5) and Ad.CMV-*LacZ* (moi 10). In the absence of VirD1, VirD2 can only nick ssDNA. To test the effect of only VirD2, cells were infected with Ad.RB-*Neo* (moi 5), Ad.*VirD2* (moi 5)

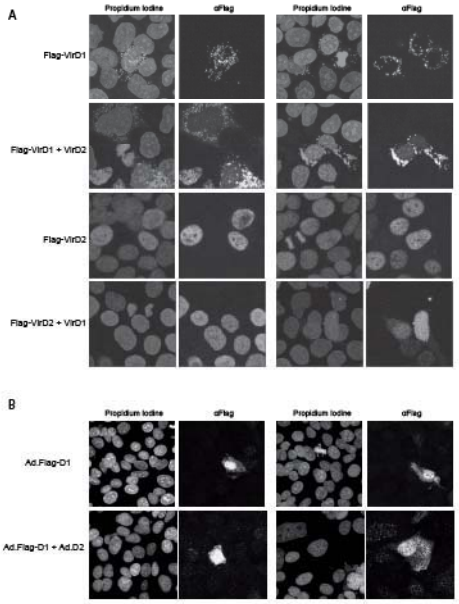


***Figure 2.*** *Immune fluorescence assays of transfected and infected U2OS cells. U2OS cells were transfected with expression vectors for Flag-VirD1, Flag-VirD2 or a combination with non tagged constructs as indicated (A). The localizations were compared to U2OS cells infected with Ad.Flag-VirD1 or co-infected with Ad.Flag-VirD1 and Ad.VirD2 as indicated (B). The flag-tagged proteins were detected by immune fluorescence confocal laser scan microscopy using mouse anti-Flag antibodies and FITC labeled rabbit anti mouse. The nuclei were stained with propidium iodine. The pictures are the projection of several Z-stacks.*

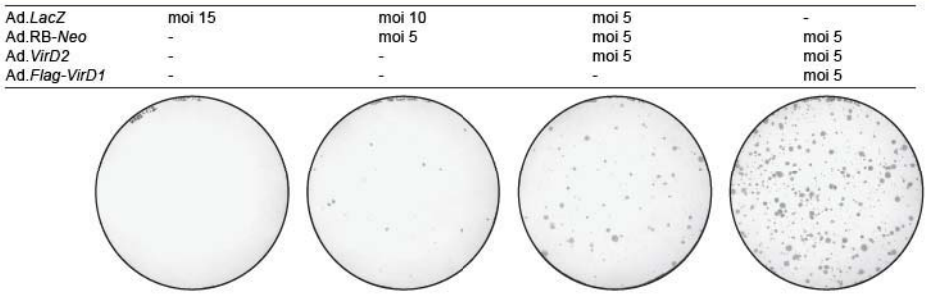


***Figure 3.*** *Formation of G418 resistant colonies. To determine integration efficiencies, U2OS cells were infected with Ad.RB-Neo and Ad.LacZ or Ad.RB-Neo, Ad.VirD2 and AdLacZ or Ad.RB-Neo, Ad.VirD2 and Ad.Flag-VirD1. In each case and MOI of 5 was used for the Ad.RB-Neo, Ad.VirD2, and Ad.Flag-VirD1 viruses. All cells were exposed to a MOI of 15, by adding Ad.LacZ, if necessary. One day post infection, the infected cells and mock treated cells from one well of a 6 well dish were split to two 15 cm dishes (1:18 dilution) in DMEM containing 400 µg/ml G418. Two-days post transfection the G418 concentration in the U2OS cultures was raised to 800 µg/ml. Five days post infection, all cells in the mock infected U2OS dish died. The cells on the 15-cm dishes were fixed and stained with bromophenol blue twenty-eight days post infection.*

and Ad.CMV-*LacZ* (moi 5). The activation of integration by both Flag-VirD1 and VirD2 was tested by infecting cells with Ad.RB-*Neo* (moi 5), Ad.*VirD2* (moi 5) and Ad.*Flag-VirD1* (moi 5). To determine the sensitivity of U2OS cells for G418, mock infected cells were included as a control. Five days post infection, all mock infected U2OS cells had died and the cells in the remaining dishes were further cultured in the presence of 800 µg/ml G418. Twenty-eight days post infection cells were fixed and the colonies were stained using bromophenol blue and imaged (Figure 3A).

Co-delivery of Flag-VirD1 and VirD2 increased the integration efficiency in U2OS cells over 60-fold (see table 1). When only VirD2 was administered integration efficiencies still were 12-fold higher despite the requirement of VirD1 on double-stranded DNA. Similar results were obtained in with Hep2 cells (data not shown).

*Table 1: Effect of VirD1 and VirD2 on the integration efficiency.*

| Viruses used | Mean Number of G418-resistant colonies | Integration frequency |
|---|---|---|
| Ad.RB-Neo, Ad.Flag-VirD1, Ad.VirD2 | 4150 | 1: 200 cells |
| Ad.RB-Neo, Ad.LacZ, Ad.VirD2 | 374 | 1: 1020 cells |
| Ad.RB-Neo, Ad.LacZ | 66 | 1: 12500 cells |

*Before infection, U2OS cells were counted and infected (moi=5 for each virus).*
*The integration frequency was calculated by dividing the number of G418-resistent colonies that were formed with the total number of cell plated.*

To test the genomic stability of the integration constructs, several monoclonal G418 resistant Hep2 cell lines were isolated and cultured for 65 days in the presence or absence of 400 µg/ml G418. Subsequently genomic DNA was isolated from each of the clones, and used for Southern analysis, with the *neo*-gene as a probe. The integration cassette was detected with a *nptII* gene-specific probe by standard Southern analysis (Figure 4A). When comparing *Hin*dIII fragments containing the $Neo^R$ gene between monoclonal cell lines cultured on G418 none of the 14 cell lines grown in the absence of G418 selection lost their integrated $Neo^R$ gene. However, in four of these cell lines (1.1, 1.6, L.4 and L.5) a rearrangement had appeared. In two cell lines (1.12 and 1.13) two distinct bands were visible of which one band was lost in the cell lines cultured with G418. So T-DNA integration in U2OS cells did not result in massive rearrangement after selection for G418 resistance.

To test if the $Neo^R$ gene would be silenced in the absence of G418 selection, monoclonal cell lines were cultured for 69 days in the

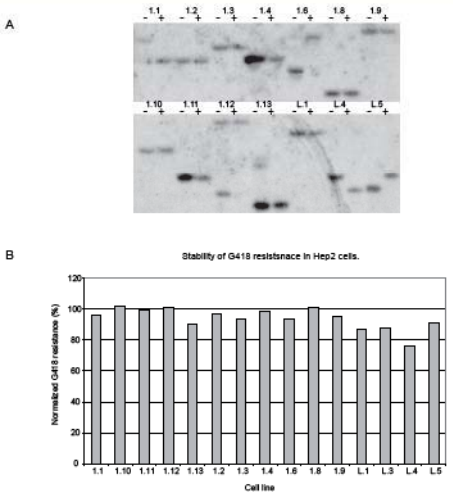


***Figure 4.*** *Stability of G418-resistant colonies. A) Hep2 monoclonal cell lines have been isolated twenty days post infection. These monoclonal cell lines were cultured for 65 days in the presence or absence of 400 µg/ml G418 and genomic DNA was isolated. The genomic DNA was digested with HindIII, size separated, blotted and hybridized to a radio labeled Neo probe. The 1.x lanes contain monoclonal lines derived from Ad.RB-Neo/Ad.Fag-VirD1/Ad.VirD2 infected cells and the L.x lanes are monoclonal lines derived from Ad.RB-Neo/Ad.LacZ infected cells. B) Monoclonal Hep2 cell lines were cultured for 69 days in the absence G418. Subsequently, all cell lines were 2400 times diluted and cultured in the presence of 400 µg/ml G418 for 11 days to select for the outgrowth of G418 resistant colonies. The number of G418-resistant colonies was counted and compared to the number of colonies in control cultures maintained in the absence of G418. The relative number of G418 resistant colonies was normalized against the number of colonies formed in the control dishes.*

absence or presence of 400 µg/ml G418. Subsequently, all cell lines were 2400 times diluted and cultured in the presence or absence of 400 µg/ml G418 for 11 days after which colonies were counted (Fig. 4b). The colony numbers in cell lines receiving G418 and those that did not during 69 days were very similar suggesting that the vast majority of the cells still expressed the neomycin-resistance marker (i.e. 90-100% of the Ad.RB-*Neo*/Ad.*Flag-VirD1*/Ad.*VirD2* infected cells.) For the Ad.RB-*Neo*/Ad.*LacZ*/ Ad.*VirD2* cells the stability was slightly lower (75-90%).

## Discussion

Human Ads are efficient vectors for gene delivery to a wide variety of cells. However, transgene expression is usually transient. This is due in part to loss of the transgene after mitotic cell division. To prevent post-mitotic loss of the transgene, the transgene should be integrated into the host cell genome. Here were evaluated the use of the *Agrobacterium tumefaciens* T-DNA system for enhanced specific integration of the transgene cassette after adenovirus mediated gene transfer.

This bacterium encodes multiple proteins that act in concert to excise and transfer a single-stranded T-DNA to plant cells. In plant cells, the T-DNA is transferred to the nucleus and integrated into the plant genome. When a plasmid encoding the T-DNA is introduced into plant cells, only the *A.tum.* proteins VirD1 and VirD2 are required for efficient T-DNA integration. Integration occurs via an illegitimate recombination process driven by host-cell enzymes. The low mechanistic complexity of T-DNA synthesis and the dependence on host proteins for integration may explain the observation that *A.tum.* can transfer genes into a wide variety of host species. This prompted us to study the feasibility of using the *A.tum.* system for integration of the transgene cassettes after their adenovirus-mediated transfer.

The flag-tagged VirD1 (Ad.*Flag-VirD1*), *wt* VirD2 (Ad.*VirD2*) and the T-DNA encoding Ad.RB-*Neo* were cloned into rAdVs. After infection the viral DNA is delivered in the host nucleus. Therefore, both Flag-VirD1 and VirD2 are required in the nucleus for the generation of T-DNA. Since only VirD2 contains a NLS, lack of VirD1 in the nucleus might limit T-DNA synthesis. For VirD1, two mechanisms could facilitate nuclear localization. First, the 18 kDa VirD1 protein is small enough to be able to diffuse through the nuclear pores. Second, Relic and colleagues showed nuclear import of GFP-VirD1 in the presence of VirD2 using fluorescence microscopy and VirD1/VirD2

interaction by coimmunoprecipitation assays[24]. We tested the localization of VirD1 by transfection and infection of human osteosarcoma cells (U2OS). In accordance to the VirD1-GFP data from Relic, we detected Flag-VirD1 in the cytoplasm in transfected cells. However, whereas Relic found the protein to be diffusely present, we could detect Flag-VirD1 mainly in discrete bodies. The formation of the bodies could be the result of protein aggregation upon over-expression. Alternatively the GFP fusion used by Relic may affect the distribution.

We also tested the expression pattern of Flag-VirD1 by adenoviral delivery and could see a clear nuclear staining in the lower expressing cells. This suggests that over-expression of Flag-VirD1 could lead to the formation of cytoplasmic aggregates that block nuclear import. When Flag-VirD1 was co-transfected with VirD2, the signal of Flag-VirD1 expression was higher and more protein was located in the nucleus. Similar results were obtained by infection of U2OS cells with Ad.Flag-VirD1 and Ad.VirD2. The distribution of transfected Flag-VirD1 was similar to that of Ad.Flag-VirD1 infected cells.

Next, we tested whether VirD1 and VirD2 could enhance the integration of T-DNA in human cells. A recombinant adenoviral genome was constructed containing a Neomycin selection cassette and a *A.tum.* right border (Ad. RB-*Neo*). The RB in the Ad.RB-*Neo* genome should be recognized by Flag-VirD1 and VirD2 resulting in a nick of the bottom strand of the RB and the synthesis of a T-DNA strand in the direction of the adenovirus L-ITR. Although *wt A.tum.* T-DNA is flanked by a left and right border. Jenn and coworkers showed that with mini Ti plasmids, either one of the borders is sufficient for T-DNA synthesis, transfer and integration. In these experiments, the RB is more processive than the left border [25]. In *A.tum.*, however, Ti plasmids with the RB deleted are avirulent while deletions of the LB have no effect on virulence [26]. Infection of U2OS cells with Ad.RB.*Neo* (moi 5) and Ad.*LacZ* (moi 10) virus resulted in G418-resistent colonies per $1.3*10^4$ cells. This is in range of the previously reported $10^{-3}$ to $10^{-5}$ spontaneous Ad integrations per cell [27]. Although VirD2 is not able to nick double stranded DNA templates in the absence of VirD1, co-infection of Ad.RB-*Neo* (moi = 5), Ad.*VirD2* (moi = 5) and Ad.*LacZ* (moi = 5) resulted in a 12-fold increase G418-resistant colony formation.

It has been reported that recombinant Ads can replicate in some tumor cells despite the lack of the Ad *E1* gene. During replication, single stranded intermediates of the Ad genome occur which might be used by VirD2 to nick at the right border. This might explain the increased integration efficiency of VirD2 alone. When U2OS cells were infected with Ad.RB-*Neo*, Ad.*VirD2* and Ad.*Flag-VirD1*, integration efficiency was induced 60-fold over background integration. Even more pronounced effects were observed in Hep2 cells (data not shown). In a similar experiment, Ad.RB-*Neo*, Ad.*VirD2* and Ad.*Flag-VirD1* infected Hep2 cells resulted in an almost confluent deck of G418-resistant colonies.

A major limitation of the *A.tum.* integration machinery is the occurrence of tandem DNA integration. These tandem integrations have been shown to lead to recombination events and silencing of the integrated T-DNA construct. The genomic stability of the T-DNA integration was studied by Southern analysis of chromosomal DNA isolated from monoclonal Hep2 lines cultured with and without G418 for 65 days. All 14 clones studied, remained positive for the Neo expression cassette. In two of the 14 lines, two integration sites were seen of which one was lost in the absence of selection. Most likely these cell lines were not monoclonal and either one of the two remained after clonal expansion. Chromosomal reorganizations were observed in 4 out of 14 cell lines. Cell

lines L4 and L5 contained Neo fragments of equal sizes. At this point, we cannot exclude the possibility that these dishes have been mixed.

To determine if silencing is a limiting factor of VirD1/VirD2 induced integration in mammalian cells, we established the rate of gene silencing by culturing Neomycin resistant Hep2 clones with and without G418 for over two month. In the presence of G418, cells are selected against DNA silencing while cells cultured without G418 can undergo DNA methylation, and silencing, or genomic reorganization. Monoclonal cell lines cultured without G418 for 69 days were diluted and cultured on G418 selection for 11 days and compared to cells that remained without selection pressure. As a control, the same monoclonal cell lines were cultured for 69 days on G418 selection and 11 days with or without G418.

The cells that were cultured without G418 for 69 days and 11 days with or without G418 showed similar levels of colonies. The stability varied from 76 to 102 % thus we conclude that silencing does not severely limit the integration efficacy of the *A.tum.* system in mammalian Hep2 cells.

So far, the increased integration efficiency and stability of $Neo^R$ expression suggested that T-DNA integration was functional in human cells. The 60-fold increase in integration efficiency over spontaneous integration can only be attributed to the action of VirD1 and VirD2. Although no RB consensus sites have been found downstream of the Ad.RB-*Neo* genome, we can not exclude that VirD1/D2 have used an alternative nicking site in the genome. Also VirD1 and VirD2 might play an alternative role in enhancing random integration through other repair pathways. For T-DNA integration in plant it was shown that host enzymes are essential. Recently, it was found in plant that VirD2 can interact with the TATA-box binding protein and CAK2Ms, a conserved plant homolog of cyclin-dependent kinase-activating kinases [28]. Whether this interaction is essential for the host mediated T-DNA integration process is still unknown. It can not be excluded that VirD2 enhances random integration by binding to mammalian homologs.

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