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

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Contents

Thesis outline

Current generation adenoviral vectors (Ads) are not suitable for those gene therapy approaches that require long-term gene expression. This is due to their high immunogenicity and transient gene expression in fast dividing tissue. The development of gutless Ads, also known as helper-dependent Ads, is a major improvement in reducing the immunogenicity of the vector system. Gutless Ads lack virtually all viral protein-coding sequences, thus severely limiting the viral-antigen evoked cellular immune responses that may result in the elimination of the transduced cells. Safety wise, recombinant Ads are considered safe due to their inability to replicate autonomously. However, we show in this thesis that replication of recombinant Ads can be rescued by the co-infection of wild type (*wt)* Ads. In this thesis studies are described that aim at the development of a new system to prevent vector mobilization. Though at its present state not directly applicable, this system could also potentially be used for the production of gutless Ads devoid of helper Ad contamination.
To improve efficacy of the Ad vector in dividing tissue we also studied two integration systems for their applicability in Ads. Overall, the experiments described in this thesis aim at generating safer vectors that should result in prolonged transgene expression due to lower immunogenicity and genomic integration.

In the first part of this thesis (Chapters **2**, **3** and **4**) we explored a new strategy to produce Ads that are replication-deficient and cannot be mobilized by *wt* Ads. Our strategy is based on the sequence specificity of replication initiation of human Ads. All human Ads start their genomes with 5'-cytidine residue. The Ads replication machinery is only able to start replication *in vitro* with dCTP as substrate. This is the result of an intrinsic specificity of pTP/Pol complex for binding dCMP as the first nucleotide. By changing the sequence at the replication-initiation sites in the HAdV5 ITRs (i.e. changing guanine residues at nt 1, 4 and 7 of the template strand to cytidine), replication in the presence of HAdV-5 pTP could be blocked.
The rHAdV-5 that have so far been used in clinical studies are replication deficient due to deletion of their *E1* region. When rHAdV-5 is co-infected with *wt* HAdV5, the *wt* HAdV can complement the *E1* deletion and thus can mobilize these rAd vectors. We show in chapter **2** that rHAdV-5-vector mobilization is not restricted to *wt* HAdV-5, but that *wt* HAdVs of other species (formerly subgroups), too, can mobilize this vector. Vector mobilization is undesirable since it can result in unpredictable side effects, especially when transgenes are used with a small therapeutic window. Use of mobilization-resistant Ads (mrAds) could potentially prevent this problem.
For the generation of these mobilization-resistant Ads a suitable production system must be made available. We searched for Ads that have ITRs that differ from the consensus HAdV ITR and looked for changes in the replication-initiation proteins that could explain this altered specificity for the ITR sequences. In chapter **3** we analyzed a series of Ad ITR sequences and confirmed that most Ad species initiated replication by coupling dCMP to pTP. The only exception is the Fowl Adenovirus 1 (FAdV-1). Some FAdV-1 isolates, i.e. PHELPS and KUR, contain guanine residues at the 5' end of the ITR. Clearly, PHELPS pTP/Pol should be able to initiate replication with dGTP residues as substrate, a feature not present in HAdV-5 pTP/Pol complex as evident from in vitro studies. The highly similar FAdV-1 isolate OTE, resembles the conventional ITR and initiates replication with dCTP. We

studied whether PHELPS had a changed specificity (i.e. can only initiate with dGTP), or a relaxed specificity (i.e. can initiate with both dGTP and dCTP) by comparing pTP and Pol sequences of PHELPS with OTE. We determined the coding sequences of OTE *pTP* and *Pol*, and performed *in vitro* replication assays. From our data we concluded that the FAdV-1 members have relaxed, rather than changed its nucleotide specificity for replication initiation.

In chapter **4** we describe the generation of mdHAdV-5 vectors with mutated ITRs (i.e. guanine instead of cytidine residues at nt 1, 4 and 7 of the top-strand). Transfection of plasmids encoding the mrAd genome on 911 producer cells did not result in the production of virus. Transfection of mrAds where the ITRs were changed back to *wt* ITR did result in virus production. This confirms that HAdV-5 pTP is not able to replicate on ITRs containing cytidine residues at the initiations sites of the template-strand. To propagate these mdHAdV-5 vectors, changes in the HAdV-5 pTP are required. We generated a FAdV-1/HAdV-5 hybrid pTP (H2pTP) by replacing a region surrounding the nucleotide-binding site of the HAdV-5 pTP gene with the corresponding region of the PHELPS pTP gene and generated a 911 cell line stably expressing this H2pTP (911-*H2pTP*). Transfection of mrAdV5-GFP plasmid on 911-*H2pTP* did result in the generation of virus indicating that over-expression of H2pTP could rescue the mutations in the ITR of mrAds.

The second part of this thesis is focused on the generation of integrating adenoviral vectors to prolong transgene expression in dividing cells. Chapter **5** describes the exploration of a plasmid-based minimized bacteriophage Mu integration system for transgene integration. Transfection of the Mu genes with the integration cassette did result in an increase of integration over background, albeit with low efficiency.

In chapter **6** we generated rAdV expressing *Agrobacterium tumefaciens* VirD1 and VirD2 genes to study 'agrolystic' integration in human cells. In this preliminary study we found a 60 fold increase in integration efficiency of Ads containing the integration cassette when both VirD1 and VirD2 were expressed in the same cell. When studying the integration sites, we found that integration did occur via an alternative VirD1/VirD2 enhanced mechanism.

In chapter **7**, we summarize the pros and cons of integrating vectors and discuss their safety.

CHAPTER 1

Introduction

The Adenovirus and its genome

Adenoviruses (Ads) are small protein particles containing DNA encoding its viral genome. Ads can infect susceptible cells and when these cells support Ad replication, the Ads will replicate and spread to neighboring cells. Ads were initially discovered in a study to culture human epithelial cells by Rowe and colleagues in 1953 [1]. The first indications of the virus pathogenicity were obtained in 1954 from febrile military personnel suffering from a variety of respiratory illnesses [2,3]. Ads have since shown to predominantly cause acute repertoire diseases, intestinal infections and conjunctivitis and are in general mild in immune-competent man. However, in transplantation recipients and immune-compromised patients, Ad infection can be lethal [4-7]. Considerable panic was caused by the discovery that inoculation of newborn hamsters with Human Adenovirus type 12 (HAdV-12) [8-10] causes tumours, which was associated with abortive infections. In rodents, most other Ad serotypes are unable to induce tumor formation [9,11] . Transformation of rodents by HAdV-12 was attributed to expression of the viral E1A gene. With the availability of tissue culture techniques, both tumorigenic [12] and non-tumorigenic Ads [13] appeared to be able to transform cells *in vitro*. So far, no human Ads have shown to cause tumors in humans.

In July 2002 a new classification of the Ads has been approved by the International Comity on Taxonomy of Viruses (ICTV), and now consists of genii *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus* and *Siadenovirus*. The human Ads belong to the *Mastadenovirus* genus, which consists of 6 type species (HAdV-A, B, C, D, E and F). Currently, 51 different human serotypes have been identified and genotyped in these 6 groups. The work in this thesis is based on the Human Adenovirus serotype 5 (HAdV-5), which belongs to the HAdV-C type species. Two other viruses used in this thesis are the Fowl Adenovirus-1 (FAdV-1, also known as CELO) isolates PHELPS and OTE. These isolates belong to the FAdV-A type species of the *Aviadenovirus* genus[1].

The HAdV is a non-enveloped virus of 70-90 nm in diameter formed by the icosahedral capsid. This capsid consists of viral encoded proteins only, which ensure cell entry, endosomal escape, and the delivery of its genome into the host nucleus. In addition, it encloses the viral DNA and protects it from degradation during these processes

The viral genome encodes four early transcription units (*E1*, *E2*, *E3* and *E4*), two delayed early (*IX* and *IVa2*) and one late transcription unit. The late transcription unit encodes five mRNAs (*L1* to *L5*). Most of these mRNAs encode capsid proteins or proteins that play a role in capsid assembly and genome packaging (Review: Pettersson U, Roberts RJ. Adenovirus gene expression and replication: A historical review. Cancer-Cells 1986;4:37–57). In addition to the late proteins, the delayed-early pIX protein is also associated with the capsid. The other delayed early protein (pIVa2) is involved in both the regulation of *E2* transcription and Ad genome packaging.

The early transcription unit 1 (*E1*) is the first to be expressed after infection and encodes the E1A and E1B proteins. E1A and E1B proteins activate transcription of *E2* and *E4* and stimulate the host cell to enter S-phase. Expression of E1A induces apoptosis of the host cell, which is inhibited by E1B and one of the *E4* proteins (E4 ORF 6). Other functions of *E4* proteins are cell cycle regulation and apoptosis induction in a later stage of infection. The *E3* transcription unit encodes proteins involved in suppression of the host antiviral responses. This region is not essential for vectors produced *ex vivo*. Besides *E3*, two RNA Polymerase III transcribed RNAs, called VA-I and VA-II RNA, function as suppressors of the host antiviral response by inhibiting protein

1 International Comity on Taxonomy of Viruses. http://www.ncbi.nlm.nih.gov/ICTV/ http://www.ictvdb.iacr.ac.uk/Ictv/fs_adeno.htm

kinase R (PKR) activation. Finally, the *E2* transcription unit encodes two regions, *E2A* and *E2B*. The E2A and E2B proteins are involved in the replication of the viral genome, and will be discussed in more detail below.

Gene Therapy using Adenoviral vectors

The concept for gene therapy was already raised in 1972 after the observation that viral DNA could stably integrate in the host genome [14-17]. In 1973, Graham and van der Eb efficiently introduced foreign DNA into mammalian cells by treating the cells with precipitates of DNA and calcium phosphate [17]. This socalled calcium phosphate transfection method made it possible to introduce Adenoviral DNA into eukaryotic cells [18,19] to identify fragments of the viral genome that are capable of cell transformation [20,21]. Such a cell line, created after transfection of sheared HAdV-5 DNA, is the transformed Human Embryonic Kidney (HEK) cell line 293 [22]. Adenoviral mutants in the E1 region were shown to be replication and transformation defective but replication could be rescued by propagating these vectors on 293 cells [23,24]. These discoveries made it possible to generate viruses that are unable to replicate autonomously. Eventually, further developed versions of these crippled vectors were used as gene delivery vehicles.

The first generation recombinant adenoviral vectors lack the *E1* and *E3* regions. These vectors can be used to deliver approximately 8 kb of exogenous DNA to susceptible cells. A disadvantage of these first generation vectors is the relative strong immune response they trigger, resulting in the eradication of the transduced cells. There are three main factors that contribute to this immune response. The strongest immune activator is the therapeutic transgene which, in most therapeutic approaches, will be a novel antigen. Secondly, low-level expression of viral proteins does occur despite removal of the *E1* genes [25,26]. These residual levels of expression can be enough to trigger a cellular immune response [27-33]. Thirdly, the capsid proteins of viruses that entered cells are also able to activate the immune system [55].

The induction of the antiviral immune response can be partially blocked by retaining the *E3* region in the vector [34]. Most likely, multiple E3 functions are required since the expression of only E3-14.7K or E3-gp19K is not sufficient to prolong transgene expression *in vivo* [35,36]. In contrast to the data obtained by Schowalter and colleagues, expression of E3-gp19K was shown to prolong transgene expression in B10.HTG mice [37]. The E3-gp19K was found to have different affinities for various MHC class-I haplotypes, which might explain the differences in transgene prolongation found by Schowalder and Bruder. More recently, a report appeared where anti-inflammation activity was shown for the E1 proteins [38]. This role for E1 in immune suppression is probably important as an early defense against the immune system and will be taken over by other viral proteins, like E3 and the VA RNAs, in later stages of infection.

The second and third generation viruses are characterized by additional modifications and deletions in the *E2* and *E4* genes resulting in reduced expression of the remaining viral genes [39-43]. In general, these vectors result in longer transgene expression. However, like the first generation vectors, they cannot fully prevent a gradual decrease of transgene expression over time.

Most promising results are to be expected from recombinant viral vectors from which all viral genes have been removed. The so-called "gutless", "helper-dependent" or "minimal" adenoviral vectors only contain the Inverted Terminal Repeats (ITRs) and the adenovirus packaging signal [44-48]. Gutless Ads can incorporate 35 kb of exogenous DNA. So far, results obtained with these vectors seem promising. While first generation vector rarely exceeded transgene expression over

one month in immune competent animals, transgene expression with gutless Ads of over ten months have already been reported [49-52]. On the downside, transgenes driven by certain artificial viral promoters are less active in gutless vectors. The reintroduction of *E4* in gutles vectors restored promoter activity to levels comparable with first generation vectors [53]. Alternatively, this effect can be overcome by the usage of non-viral, tissue specific promoters.

Limitations in Adenovirus mediated gene therapy

Currently, third-generation Ads are efficient gene-delivery vehicles that give rise to a transient expression of the delivered gene. For long-term therapeutic effects, third generation vectors seem less suitable for reasons mentioned above. The current limitations can be roughly separated into 3 groups; vector targeting, duration of transgene expression and immune response.

The widely used HAdV-5 transduces liver and lung very efficiently when administered systemically. Therefore, Ads are most efficiently used in therapies targeting these organs. Yet, for therapeutic approaches where specific cell types need to be transduced, improved targeting strategies are required. These strategies may also be used to prevent transduction of unwanted cells, e.g., antigen-presenting cells. Specific targeting can be achieved by modification of viral proteins that bind the host cell. Alternatively, expression of the transgene may be targeted to specific cell types using cell-type specific promoters.

Insertion of the transgene in the host improves the vector efficacy by prolonging gene expression, since integrating vectors are no longer lost after cell division. Transgene integration in the host genome, and especially targeted integration, can prevent the loss of the therapeutic gene resulting in prolonged gene expression.

Increasing the administered viral titer does improve the therapeutic effect transiently; though, it also enhances the chance to evoke an immune response. Both the immune response against the transgene, which is usually foreign to the patient, as well as the immune response against the Ad vector itself are major problems that need to be solved. The immune response against the gene of interest is one of the most challenging problems and so far no strategies have been shown to be sufficiently adequate in clinical trials. One promising approach is the use of the Gly-Ala repeat of the Epstein-Barr EBNA-1 protein as a *cis*-acting sequence to block MHC class 1 presentation. Repeats fused to LacZ have been shown to block LacZ specific CTL recognition, although the therapeutic efficacy remains to be established [54].

The immune response directed against the viral vector is a more recognized and probably more severe problem. Reports have indicated that an immune response can be triggered by residual viral gene expression, which does take place in first-generation vectors. This response can be severely lowered by the use of vectors that contain additional deletions in *E4* and/or *E2A*, since these deletions lower residual viral replication and gene expression. The use of Ads stripped of all viral genes, the so-called gutless Ads, should solve this problem. Unfortunately, immune responses can also be triggered by the viral capsids as well, indicating that the introduced viral proteins somehow can be processed to trigger an immune response [55]. Complete manipulation of the viral capsid to lower immunogenicity is probably not feasible, so more efficient vectors are required that can be introduced at lower titers.

Propagation of gutless Ad vectors

While gutless Ads should be less immunogenic, the production of these vectors is more challenging than first generation Ads. For the propagation of gutless Ads, all viral genes need to be provided *in trans*. The generation of cell lines stably expressing

these viral proteins is not a feasible option since constitutive expression of some Ad proteins is toxic to cells. So far, no data has been reported for the generation of helper cell lines in which all essential Ad genes are stably integrated and controlled by an inducible promoter. Yet, an Epstein-Barr based amplicon system expressing E2 and E4-ORF 6 under the inducible tetracycline promoter has been described [56].

Currently, the standard technique to propagate gutless virus involves the coinfection of a helper virus. This helper virus can be a *wt*Ad or an *E1/E3* deleted Ad. In the latter case, the use of an E1 complementing helper cell line is required. Coinfection with helper virus results in the expression of all viral genes required for the replication and packaging of the viral genome into infectious particles. One major drawback of this coinfection strategy is that both gutless- and helper Ads are replicated and packaged [57-59]. Initially, the genome of the gutless Ads were deliberately kept smaller in order to remove the helper virus by density gradient fractionation. Even so, with this method not all helper Ads could be removed and the remaining 1% helper Ad contamination remained a problem. The use of these vector batches would still induce an inflammation reaction due to viral gene expression of contaminating helper Ads.

To prevent contamination of gutless Ad batches with helper Ads, the packaging signal of the helper Ads was modified to prevent packaging of these vectors. Surrounding the Ad packaging signal, Lieber and colleagues cloned LoxP sites [60]. The LoxP sites are recognized by the Cre enzyme which leads to the excision of the intervening packaging signal. In combination with a helper cell line expressing Cre, helper-virus contamination has been decreased to less than 0.01% [61]. This elegant approach still has some limitations that need to be addressed in order to make optimal use of the advantages of gutless vectors.

The first problem is homologous recombination between helper and gutless Ad due to the homology up- and/or downstream of the packaging signal. This recombination results in the loss of one or both LoxP sites preventing packaging-signal excision and resulting in the packaging of the helper-virus genomes. Alterations in the packaging signal of the helper virus reduced the frequency of recombination events with the gutless vector thus diminishing helper contamination [62].

Secondly, removal of the packaging signal is fully dependent on the activity of the Cre enzyme. Since this strategy is relying on enzyme kinetics, it cannot fully prevent helper contamination without additional strategies [63].

Finally, a minor problem is caused by the packaging of helper genomes that still lack the packaging signal [64]. Although these vectors do not contain the growth advantage of helper Ads containing a non-excisable packaging signal, they are able to trigger an immune response when administered to immune competent patients. The use of third generation Ads as helper virus might help in lowering the toxicity of the contaminating helper Ads.

New strategies to produce helper-free gutless Ads

A first approach to develop a gutless Ad production system without contaminating helper Ads has been described by Cheshenco and colleagues [65]. They included a *E1*, *E3*, and packaging signal-deleted Adenoviral genome, flanked by LoxP sites, into the AcMNPV Baculovector. The AcMNPV vector is able to infect mammalian cells but does not replicate or express viral genes [66-70]. Infection of this Baculovector into 293-*Cre* cells resulted in the excision and circularization of the Ad genome by the Cre expressing helper cell line. The authors hypothesized that the internal ITRs could be used as replication initiation sites, as was also described by others [71,72]. This would result in replication competent but packaging

deficient Ad genomes capable to act as helper viruses for the generation of gutless vectors. The relative high titers of Baculovirus (moi = 2000) required for efficient gutless vector mobilization did suggest that the circularized helper Ads were not efficiently replicating. Furthermore, homology between the 293 cell line and the helper Ad genome resulted in the formation of Replication-Competent Adenovirus (RCA). The formation of RCA is a known problem during the propagation of Ads in 293 or 911 helper cells but can be prevented by use of the PER.C6 helper cells in combination with matched vectors [73].

A completely different approach was suggested by Zang and colleagues [74]. Viruses deleted for *L1 52/55K* were used to study the role of L1 52/55K during the early stages of infection. Interestingly, this mutant resulted in the generation of empty capsids and had no adverse effect on viral protein expression [75]. This clearly suggests that L1 52/55K plays an important role in the packaging of viral DNA. It was already known from previous studies that L1 52/55K protein interacts with the adenoviral protein pIVa2 [76]. This protein was identified as a transactivator of the major late promoter (MLP), but L1 52/55K appeared

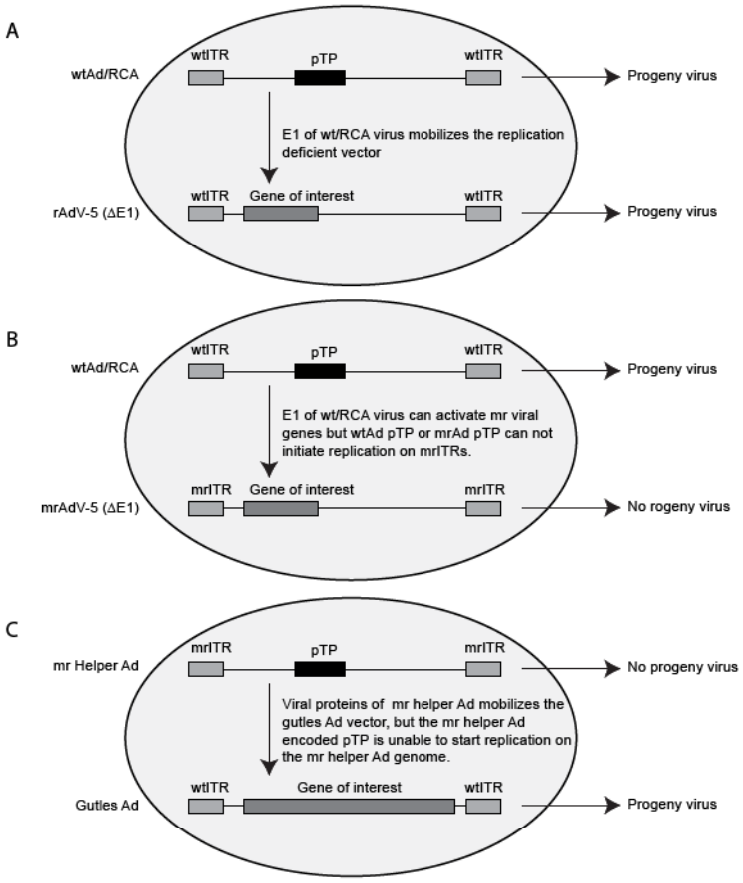

Figure 1. *Models of vector mobilization. (A) Classical mobilization of first generation Ad vectors by wtAds. The wtAds can provide E1 functions resulting in replication of both wtAd and recombinant Ad. (B) The ITRs of first generation Ads have been replaced by ITRs of mrAds. The wtAd E1 can complement the E1 deletion of mrAds but pTP is not able to initiate replication on these mutated ITRs. (C) For the production of gutless Ads, helper replication should be prevented. Therefore, the ITRs of helper Ads have been mutated to mrAd ITRs. The vector itself can not replicate but it can provide all essential genes for gutless Ads to propagate.*

not to be involved in this transactivation. Surprisingly, the Ad packaging signal shared DNA sequence homology with the MLP. These sequences in the packaging signal could be bound by the pIVa2 protein as well [77]. This suggested a role for the pIVa2-L1 52/55K protein complex in the packaging of viral genomes. When cells were infected with *L1 52/55K* deleted HAdV-5 and coinfected with *wt*Ads of other serotypes, it appeared that HAdV-7, HAdV-12 and HAdV-17 were unable to complement the L1 52/55K defect in HAdV-5. By generating HAdV-7 vectors containing HAdV-5 ITRs and packaging signal, Zang and colleagues showed that the virus replicated and expressed viral proteins but was unable to package its genome [74].
This system provided a possible method to generate helper Ads that are unable to be packaged in the absence of HAdV-5 pIVa2 protein and thus provides a strategy to generate HAdV-7 gutless vectors without HAdV-5/7 hybrid helper virus contamination.

In the first part of this thesis we describe experiments that aim at generating rHAdV-5 vectors that can not be mobilized by *wt*Ads anymore. By introducing specific mutations in the ITRs, rHAdV-5 vectors were generated that are not recognized by the *wt*Ad replication complex. Furthermore, by modifying the proteins involved in recognizing these mutated ITRs, we created a new specificity that resulted in vectors that we called mobilization-resistant Ads (mrAds). These vectors are an important safety improvement over current generation Ads since their replication can not be rescued by wtAds. Ultimately, this system might be suitable for the production of gutless Ads where the helper Ad is replication deficient but does deliver all viral genes required by the gutless Ad for replication, packaging and release. Vector mobilization and our mechanism to prevent mobilization is depicted in figure 1 and mechanistically in figure 2. The adenoviral replication machinery will be discussed in the next section.

Viral DNA replication

DNA viruses have different strategies to replicate their genomes. All viruses need to conform to 5' -> 3' DNA synthesis and thus need a 3'-OH group to start elongation. Viral replication should be efficient and produce replication-competent DNA copies since these progeny DNA copies are used as templates in subsequent rounds of infection. These requirements produce particular challenges for the evolutionary development of linear DNA viruses. Replication of linear DNA molecules without a specialized mechanism to protect the integrity of their terminal sequences during each round of replication, results in loss of terminal sequences [98]. A well-known example is the telomere sequence that is shortened each time the genome duplicates in cells without Telomerase activity. To prevent loss of terminal repeats, some linear DNA viruses, like the Epstein-Barr virus, convert to a circular DNA form during the replication cycle introducing an internal replication origin. Other viruses like the Adeno-Associated Virus, a single-stranded DNA virus, form hairpin structures in their terminal sequences. Hairpin formation in the 3' terminus results a single stranded template strand with a partial double- stranded initiation site containing a 3'-OH that is used by the DNA Polymerase as initiation site for replication. This strategy requires a specialized nicking enzyme that nicks the double stranded 3' terminal template.
Thirdly, some linear DNA viruses, like bacteriophage PRD1 and Ads, employ a protein primer for replication initiation. Protein priming is not restricted to DNA viruses but is also used in some RNA viruses such as Poliovirus. In contrast to DNA and RNA primers, the protein primer does not require complementary DNA. Consequently,

it has the potential to initiate replication on the last 3' nucleotide of the genome. However, both bacteriophage PRD1 and Adenovirus do initiate replication upstream of the 3' terminus and have developed alternative mechanisms to maintain the integrity of the termini known as "Sliding back" [99] and "Jumping back" [100] respectively.

Adenovirus Origin of Replication

All Ads contain Inverted Terminal Repeats (ITRs) on both ends of their genome. The length of this repeat can differ between species and serotypes but the functional organization is the same. The first functional element is the 'repeat region' and starts at the extreme end of the genome. The repeat region is composed of multiple 2, 3 or 4 base pair repeat units, dependent on the Ad serotype, and is a direct consequence of the "Jumping back" replication mechanism that these Ads employ (as described below and figure 2).

The next functional element, located

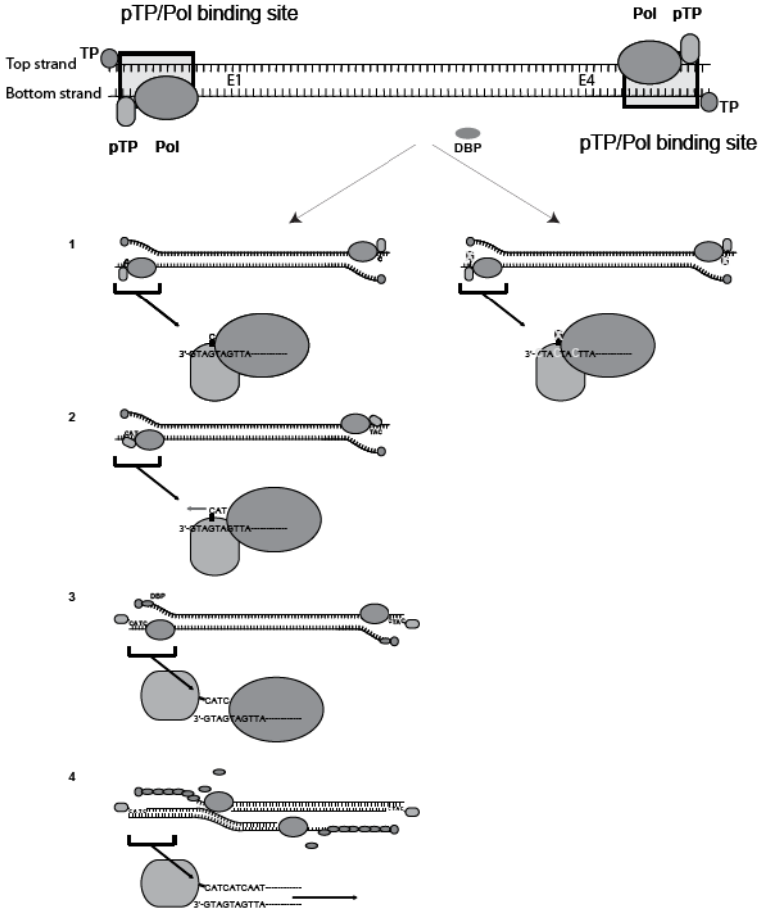

Figure 2. *Schematic presentation of the steps involved in Adenovirus genome duplication. At the top, the Ad genome is presented with the replication initiation sites containg the TP, pTP Pol and DBP proteins highlighted. In the left column, the specific steps; initiation (1), elongation (2) and (3) and second strand synthesis (4) are described in the main text.*
In the right column, a model of mdAd replication initiation is shown. (See Chapters 3 and 4)

downstream of the repeat region, is the pTP/Pol binding domain (or 'core region'). This functional domain of approximately 10 bp is conserved between all Ad species. During replication initiation, this domain is bound by the pTP/Pol replication initiation complex, protecting bases 8 to 17 in a DNaseI protection assay [101]. Comparative binding studies revealed pTP as the most dominant factor for binding the pTP/Pol domain [102] although purified Pol has also been shown to bind the pTP/Pol binding domain [101]. Mutations in the pTP/Pol binding domain resulted in a strong inhibition of replication initiation *in vitro* [103-107], while mutations of nucleotides 12 to 16 hardly changed replication initiation efficiency, at least for Human Adenovirus type 4 (HAdV-4) [108].

Downstream of the pTP/Pol binding domain is the auxiliary region located [109]. In HAdV-5, this region contains binding sites for the human transcription factors NF1 [110-112] and Oct-1 [113,114]. This region of approximately 29 bp is not essential for *in vitro* replication initiation. *In vivo*, only NFI seems essential for replication [105] while Oct-1 enhances replication [105,115-117]. Surprisingly, only the DNA-binding domains (DBD) of both transcription factors are required for the replication stimulation. Although NFI and Oct-1 interactions are mostly weak, replication can be stimulated up to 200 fold *in vitro*. The NFI-DBD can activate replication in vitro 2-50 fold, while the DBD of Oct-1 (POU domain) activates replication 6 to 8 fold. The fold activation of both transcription factors is dependent on pTP/Pol concentrations [118,119]. Since these transcription factors are host specific, the auxiliary region is not conserved between Ad species. Differences exist even within the HAdV family. While some serotypes lack the presence of the NFI site (HAdV-4) others lack both sites (HAdV-10). It is not known whether these viruses employ other mechanisms to stimulate replication.

Steps in Ad DNA replication

Pre-Initiation Complex formation and DNA binding. The Ad replication protein pTP and Pol can form a stable heterodimer *in vitro* in the absence of other proteins or DNA templates [101,102]. *In situ* pTP/Pol association has been shown using HeLa lysates isolated during the early onset of Ad infection [120]. Literature on DNA binding by the single proteins is confusing. While Chen and colleagues reported that pTP was only able to bind to the origin in the presence of Pol [121], Temperly and Hay reported DNA binding activity of both pTP and Pol [101]. They also mapped the first 180 aa of pTP as the region responsible for binding. The region of Ad Pol contacting the DNA was mapped in the N-terminal part [122]. Binding of the pTP/Pol complex to the Ad origin seems stronger than the binding of both pTP and Pol together. Furthermore, binding of the pTP/Pol complex to the origin is enhanced by the cellular transcription factors NFI and Oct-1 [123,124] of which only the DBD is required [125,126].

The NFI enhanced binding of the pTP/Pol complex to the origin can be explained by recruitment of the complex to the correct position on the DNA. In support, NFI has been shown to stabilize the binding of the pTP/Pol complex to the DNA by binding to Pol, which is dependent on the pTP/Pol concentration [119,121,127,128]. Furthermore, it has also been shown that binding of NFI to the auxiliary region induces structural changes in the DNA [129]. Therefore, enhanced DNA binding of the pTP/Pol complex to the origin due to conformational changes in the DNA should also be considered. Support for this hypothesis was delivered by Dekker and colleagues, who demonstrated that the recruitment of the pTP/Pol complex to the origin by a NFI-DBD mutant (aa 1-75) was enhanced although this mutant was unable to bind to the pTP/Pol complex in a GST-pull-down assay [127]. So far, there are no reports that NFI induces conformational changes in

Pol that result in enhanced DNA binding. Kinetics studies revealed that NFI does not affect the intrinsic activity of pTP/Pol to bind the first nucleotide [128].

The function of the Oct-1 transcription factor in Ad replication is in many aspects similar to NFI. Like NFI, Oct-1 is able to recruit the pTP/Pol complex to the pTP/Pol binding domain in the ITR via a direct interaction with pTP [113,114,130]. The enhanced DNA binding of the pTP/Pol complex to the origin is depended on the pTP/Pol concentration, where stimulation was highest at low pTP/Pol concentrations [124]. Like NFI, binding of Oct-1 to the auxiliary domain also seems to induce local DNA bending [131] although a correlation between DNA bending and enhanced pTP/Pol binding was not presented. Binding of Oct-1 to pTP does not change the intrinsic activity of the pTP/Pol complex [128] suggesting that protein-protein interactions do not affect domains involved in replication initiation. However, it has not been established whether the Oct-1 induced DNA binding effect is a result of structural changes in pTP.

Furthermore, replication of DNA templates containing TP is over 20-times more efficient than replication of naked DNA [107,132-134]. This suggests an important role of TP in the replication initiation of progeny templates. DNAseI footprints suggested that TP induces structural changes in the DNA resulting in enhanced binding of the pTP/Pol complex [133]. Removal of the TP protein from the 5' strand reduced replication efficiency, which can be relieved by partial exonuclease degradation of the displaced strand. This suggests that TP also plays a role in the unwinding of the DNA origin [132,135].

Finally, the Ad DNA binding protein (DBP) indirectly enhances the binding of NFI to the DNA [136,137] further activating binding of the pTP/Pol complex to the origin.

Replication initiation. After binding of the pre-initiation complex to the pTP/Pol binding site, a dCTP residue is covalently coupled to Serine[580] of pTP [138] by the Ad Pol. This reaction is template dependent but has a strong preference for dCTP residues. Experiments performed by King and colleagues revealed that on a wtHAdV-5 template (3'-GTAGTAGTTA), the pTP/Pol complex initiates replication on nucleotide 4 or alternatively on nucleotide 7 or 1 [100]. When a 3'-GAGAGA template is used, nucleotide 5 is used as initiation site, suggesting some flexibility in the pTP/Pol active site [139]. On a 3'-GTAATAGTTA template, only pTP-C products were found and no pTP-T or other trinucleotide complexes [100].

The binding of a dCTP residue to pTP is enhanced by the Ad DNA binding protein (DBP) [140]. Enzyme kinetics studies indicate that DBP lowers the K_m of the pTP/Pol complex for binding the first dCTP most likely by inducing structural changes in the active site of Pol. However, direct interactions with Pol have not been shown [128]. Experiments performed by Coenjaerts and van der Vliet showed that the NFI-DNA and NFI-Pol interaction is lost as soon as the PIC/DNA complex is incubated with dCTP, dATP or dTTP *in vitro* [141]. Since these nucleotides are used for the formation of pTP-CAT, structural changes in Pol or DNA lead to dissociation of NFI. Whether the NF-I/Pol interaction or Oct-I/pTP interaction also affect substrate specificity is not known.

Replication elongation. After pTP binds the first dCMP, Pol elongates the pTP-C complex further to a pTP-CAT complex. After formation of the pTP-CAT, the interaction between pTP and Pol is partially lost [139] and the complex is jumping back to position 1 [100]. Dissociation of pTP/Pol is not required for this step. The pTP-CAT complex at position 1 is further elongated by Pol. After the formation of a 7-nucleotide pTP complex, the dissociation of pTP and Pol is complete. At this stage the interaction of pTP with Oct-1 is still present. This interaction is lost after

the formation of a pTP complex containing at least 26 nucleotides [124].
Since DBP has dsDNA helix destabilization activity [142,143] it has been suggested that DBP is required for unwinding the Ad genome. Experiments performed by Dekker and colleagues showed that DBP is cooperative by binding the single stranded displaced strand forming multimers of DBP. This effect is required for unwinding the dsDNA helix [140]. Furthermore, DBP enhances the processivity of Pol during elongation [144]. Finally, for full-length DNA replication *in vitro*, the cellular factor NFII (a topoisomerase I) is also required [145].

Second strand synthesis. During replication, one of the strands will be displaced dependent on the origin that has been used. This results in only one fully replicated strand, leaving a DBP coated single-stranded displaced strand. Without template strand, an alternative mechanism is required to duplicate this displaced strand. Based on the homology between the left and right ITR, Daniell proposed a model of intramolecular base pairing between both ITRs, resulting in the formation of a panhandle structure [146] (see figure 3).

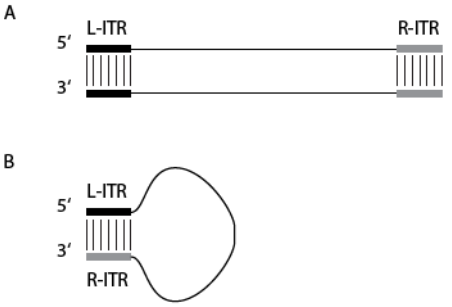

Figure 3. Different models for the Adenovirus replication origins. A) Classical model where the double stranded adenovirus genome consists of two ITRs. B) After the replication initiation from one ITR, the displaced strand can form a panhandle structure by basepairing with the ITR on the other side of the genome. In this model, the replication origin consists of a left and right inverted terminal repeat. Depending on the displaced strand, this model could correct for mutations in one of the ITRs.

Although there is no physical evidence for panhandle formation during Ad replication, several groups reported data that support such a model. Viruses with partial deletion in either one of the ITRs were able to replicate and correct this deletion [147-150]. Furthermore, genomes with non-identical ITRs were shown to be viable but progeny virus only contained one type of ITR [151]. Both observations support the model that the left and right ITR hybridize and thus deletions or mismatches can be repaired.
On the other hand, data obtained by Zijderveld and colleagues, indicates that panhandle formation is not possible with DBP coated ssDNA [151]. They showed that DBP promotes renaturation of complementary ssDNA but intramolecular renaturation is inhibited by DBP. This suggests that the observed repair of ITRs is most likely caused by inter-molecular base pairing between left and right ITRs of different single-stranded displaced strands.

Other functions of pTP

After pTP has participated in replication initiation, it is cleaved by the adenovirus-encoded protease p23. The cleavage of pTP occurs at three positions to form two intermediate forms (iTP1 and iTP2) and one Terminal Protein (TP). These processed forms lack DNA binding activity [152] and thus are not likely to be directly involved in replication initiation [153]. However, over-expression of protease p23 *in vivo* could not block adenovirus replication although incomplete processing of pTP could be argued [154]. Furthermore, in Ad Pol pull-down assays with pTP cleaved to iTP *in vitro*, both the iTP fragment as well as the N-terminal fragment were coprecipitated. This suggests that the N-terminal fragment might still associate to iTP, resulting in the formation of a complex with DNA-binding activity that can participate in replication initiation *in vivo*. The cleavage of pTP by p23 is

dependent on a cofactor generated after pVI cleavage by p23. Since iTP, p23 and the cofactor are present in viral capsids, it has been suggested that cleavage of iTP to TP occurs after encapsidation [134,155]. Disabling pTP cleavage with a temperature-sensitive p23 protease showed that pTP-containing viral genomes can still be packaged [95]. It is not known whether these particles are infectious.

A specific role for TP *in vivo* is proposed by Pombo and colleagues [156]. They found that Ad DNA replication takes place at multiple, highly organized, foci in the nucleoplasm. By pulse-chase experiments they showed that newly synthesized double stranded genomes are displaced from these foci and spread throughout the nucleoplasm where they are used as templates for transcription. Transcription takes place in specialized foci distinct from the replication foci. The role of pTP in the nucleus is further emphasized by the finding that pTP does bind to the CAD enzyme (carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase), a multifunctional pyrimidine biosynthesis enzyme. The pTP-CAD complex is localized at sites of active Adenovirus replication as determined by BrdU incorporation [157].

Colocalization of pTP with promyelocytic leukemia antigen (PML) was not detected. The functions of PML bodies are not yet completely understood but they are targeted by a fast amount of viral protein, including the Adenovirus E1A. The PML bodies are severely reorganized by the E4-ORF 3 protein, suggesting that this step is essential for viral replication. A mutant of E4-ORF 3 unable to reorganize PML bodies was also shown to have a severe defect in viral replication [158]. So disruption of PML bodies is an important step for the activation of Ad replication. Evans and Hearing also showed that relocalization of the Mre11-Rad50-Nbs1 (MRN) DNA repair complex is caused by E4-ORF 3 during early stages of infection. At later stages, Nsb1 was found at sites of active replication while Rad50 remained localized with E4-ORF 3 [159]. Whether pTP is involved in the localization of the viral genome to these replication active sites is not known.

The Adenovirus E2 transcription unit

The pTP, Pol and DBP proteins described above are all encoded by the adenovirus *E2* transcription unit (see figure 4). The organization and regulation of the HAdV-5 *E2* unit is well studied and was used in chapters 3 and 4 to serve as a reference for the identification of the coding sequence of PHELPS and OTE pTP and Pol. The HAdV-5 *E2* transcription unit contains the *E2A* and *E2B* transcription regions, which are both driven by different promoters located 1 kb apart, are alternatively spliced and utilize different PolyA signals. The transcription of *E2* genes is activated by the E2 early (E2E) promoter during early stages of infection, and the E2 late (E2L) promoter at an intermediate stage of infection. The HAdV-5 E2E promoter is located approximately 27 kb downstream of the left ITR with the coding strand located on the bottom strand of the viral genome. It contains a TATA box, two binding sites for the cellular transcription factor E2F [78] and one ATF binding site. Expression of active E2F is enhanced by E1A-13S, thus E2 expression is indirectly activated by E1A-13S [79-81]. Furthermore, the E2F transcription factor can associate with the E4-ORF 6/7 protein and cooperatively bind to the E2E promoter [82-84]. Expression of E2 proteins via the E2E promoter is suppressed in later stages during infection. This suppression is correlated to the activation of E4-ORF 4. The association of E4-ORF 4 to the cellular protein phosphatase 2A (PP2A) results in activation of PP2A. Activation of PP2A leads to the dephosphorylation of E2F which is thought to decrease the binding affinity of

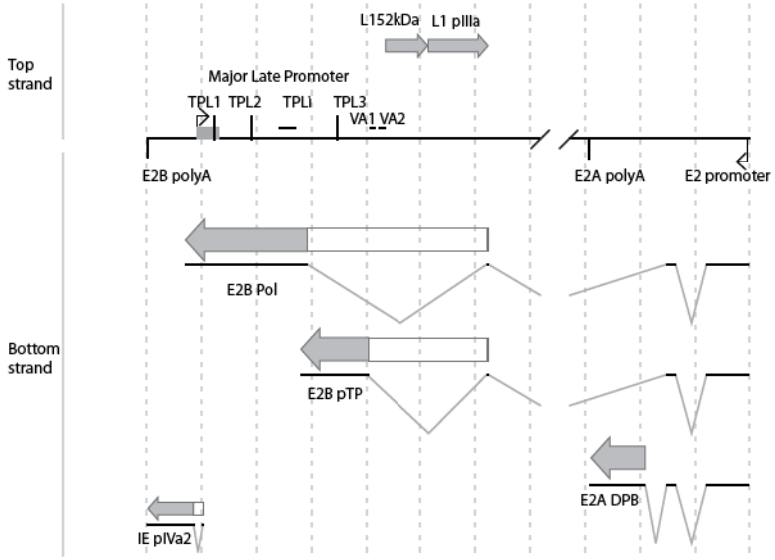

Figure 4. *Schematic presentation of genes and regulatory sequences located in the E2 transcription unit. The arrows point in the direction of transcription. Gray arrows depict coding regions while the open white region depicts intron sequences.*

E2F to DNA [85].

The E2L promoter contains a TATA box, two SP1 binding sites, and three inverted YB-1 binding sites. Two of these YB-1 binding sites are sufficient for efficient expression of E2 while the activity of the E2L promoter is inhibited by E1A-12S protein [81]. Expression of E1B-55 kDa facilitates nuclear accumulation of YB-1 protein and enhances YB-1 induced expression of E2 proteins via the E2L promoter [86]. YB-1 promotes the splicing of the E1A pre-mRNA to the 13S form [87]. Thus, besides direct binding to E2L, YB-1 might release repression of the E2L promoter by decreasing E1A-12S expression.

In addition to direct activation of the *E2* transcription unit by cellular transcription factors E2F and YB-1, chromatin-remodeling factors are likely to be involved as well. For HAdV-12, Fax and colleagues found that E2 expression was dependent on the HAT activity of the p300/CBP complex [88,89] This complex is recruited to the E2E promoter by E1A-12S. While *de novo* synthesized Ad genomes, which are transcriptionally silent, are coated with major core protein VII, transcriptionally active templates appear to be bound by histones [90]. Like on chromatin, histone acetylation could play an important role in keeping the Ad genome transcriptional active. Finally, E2 expression is also activated by one of its own gene products, the *E2A* encoded DNA binding protein (DBP) [91].

The *E2A* transcription unit encodes several mRNAs which differ in both their 5' and 3' untranslated regions (UTRs) [92,93]. The main 2.0 kb mRNA is spliced twice. The first two exons and the first part of the third exon form the 5' UTR. The major part of the third exon encodes the full DBP protein and is not spliced in the coding region. Some minor E2A mRNAs have been isolated that also differ in the 3' UTR region.

The *E2B* transcription unit encodes two major ORFs, one for the pre-terminal protein (pTP) and one for Polymerase (Pol). In contrast to the DBP ORF, these ORFs do not encode biological active proteins in HAdV-2 [94]. The E2B pre-mRNA is expressed from the same E2 promoter and shares the first

exon with the major E2A-DBP mRNA [95]. After the first exon and a 12.7 kb intron, a 187 bp exon is located. The *E2B* exon 2 contains the start codon of both pTP and Pol and nine nucleotides that are part of the coding sequence for pTP and for Pol. Exon 2 is alternatively spliced to exon 3 of pTP (pTP-exon 3) or exon 3 of Pol (Pol-exon 3) [96]. The splice acceptor in both pTP-exon 3 and Pol-exon 3 is located upstream of the major ORF [96]. Both pTP and Pol mRNAs utilize the same PolyA signal.
The switch from E2A to E2B expression is most likely regulated by splicing privilege. Lesions in the 5' UTR of *E2A-DBP* resulted in less usage of the adjacent 3' *E2A-DBP* splice-acceptor site and in an increased usage of a splice-acceptor site in the *E2B* region [97].

The complementary strand of the *pTP* and *Pol* genes does contain important coding and non-coding regions. In the 3' terminus of the Pol ORF, the *pIVa2* promoter/enhancer region and parts of the coding region of pIVa2 are located. Further upstream of the 3' terminus, *Pol* contains the complementary sequence for the major late promoter (MLP). Important for the expression of the late proteins are the MLP tripartite leader (TPL) elements. The TPL1 and TPL2 elements are located in the Pol coding region, while TPL3 is located in the pTP coding region. A fourth TPLi element, used by early and intermediate late mRNAs, is located between TPL2 and TPL3 5' of the main Pol ORF.
The intron between exon 2 and pTP-exon 3 encodes the L1-52/55K and L1-pIIIa proteins on the complementary strand. These functional elements should be considered when deletion mutants of pTP and/or Pol are generated.

By introducing modifications in the replication origins and adapting the proteins that initiate replications on these origins, specificity could be achieved that can be used to generate mobilisation resistant Ads. Recombinant Ads that can not be mobilized by *wt*Ads are safer to use in clinical settings. Furthermore, mrAds could potentially yield a gutless vector production systems that lack the generation of immunogenic helper Ad contamination, thus are expected to be more efficient. One other strategy to increase the vector efficiency is to integrate the transgene casette in the host genome. Especially in dividing tissue this requires lower initial viral doses since the transgene will be multiplied by the dividing tissue. The use of a lower viral dose should decrease the vector induced immunogenicity, while transgene integration results in prolonged expression since it is not lost after cell division. In the second part of this thesis we show the results from studies of two different integration mechanisms and their integration efficiency in mammalian cells.

Integration as a mechanism for persistence of transgene expression

There are many viruses that reside in a latent phase awaiting reactivation. Especially viruses that reside in dividing cells have highly specialized mechanisms to maintain their genomes. One of these viruses is the Epstein Barr Virus (EBV), which infects B cells of the immune system. During latency, this virus remains as an episomal replicon duplicating its genome approximately once per cell division. Other viruses have found ways to integrate their genome into the genome of the host. Awaiting reactivation, the viral genome remains in the host genome and is thus multiplied and transferred to daughter cells by the host replication machinery. Retroviruses are well known for their capacity to integrate their genome. This group of viruses transfer their genomes as single-stranded RNA molecules, which are reverse transcribed inside the host cell to form double-stranded DNA molecules that can integrate in the host genome. With lentiviruses as exception, these viruses can only integrate in dividing cells. Transcription of the integrated provirus results in the formation and spread of the *de novo* generated virus. A second example of an integrating virus is the Adeno-Associated virus. This virus relies for replication on Adenovirus proteins and is therefore dependent on adenovirus coinfection. After infection, AAV can integrate in the host cell genome with a preference for a specific locus on human chromosome 19. Here, it can reside until it is reactivated once the same cell is infected by Adenovirus or Herpesvirus. Other viruses have adopted similar strategies. For example, the Bacteriophage Mu will integrate in *E.coli* during latent infection and will be released from the *E.coli* genome by external stimuli resulting in a lysogenic phase of infection.

Adenoviruses do not have such a dedicated integration machinery. Despite some reports of Ad latency and integration associated with Chronic Obstructive Pulmonary Disease (COPD), there is no evidence that these Ads can be reactivated again [160,161]. These findings are controversial and sofar, it cannot be excluded that the Ad sequences that have been found in lung tissue are remnants of previous Ad infections.

Genomic integration of foreign DNA is not restricted to pathogens with dedicated integration machineries. Holmgren and colleagues have shown that DNA from rat apoptotic bodies, can be taken up and integrate in mouse cell cultures when cocultured [162]. In other studies, portions of mitochondrial DNA have been found to integrate in chromosomal lesion sites [163]. This clearly indicated that most cells are capable of integrating foreign DNA, although efficiencies are (yet) not sufficient to be used in a therapeutic approach.

Integration systems currently used in gene therapy approaches

Lentivirus. Lentiviruses are retroviruses that, in contrast to the other retrovirus family members, can integrate in non-dividing cells. After cell binding and entry, the ssRNA genome is converted to an integration prone dsDNA genome. For integration, retroviruses rely on the virally encoded Integrase enzyme, which is included in the viral capsid. Since oncoretroviruses are unable to penetrate the nuclear membrane, they rely on mitosis for integration. During mitosis the nuclear membrane dissolves and the host genome DNA is available to the viral pre-integration complex. Lentiviruses can penetrate the nuclear membrane and thus infect non-dividing cells as well. To enter the host nucleus, the lentivirus use Vpr, matrix protein and Integrase. The accessory Vpr protein is part of the viral capsid and localizes to the nuclear membrane where it mimics the action of importin-ß [164,165]. Though this protein plays an essential role in nuclear entry of the pre-integration complex, lack of Vpr protein in third-generation lentiviral

vectors only seem to inhibit nuclear entry in macrophages. The matrix protein is part of the Gag gene and can bind to the host nuclear import machinery. Both integrase and matrix protein contain classical nuclear localization signals.

AAV. The Adeno-associated virus is a small 4.7 kb virus containing two ssDNA copies. The replication origins are located in the 5' and 3' ITRs. Part of the 5' ITR also contains the replication-initiation site indicated by the REP-binding site. In addition to initiation of replication, REP is also involved in the site-specific integration of *wt* AAV on chromosome 19 in human cells. The integration site prevalence is most likely due to homology on chromosome 19 with the REP binding site. Recombinant AAV vectors lacking REP do integrate but lost their site-specific integration properties.

Hybrid Ads vectors. The first hybrid Ad strategy was explorered by Feng and colleagues using a retroviral genome inside a recombinant Ad [166]. Infection of the target cell with rAdV resulted in the local production of retroviral vectors containing the gene of interest. These infected cells located close to the initial site of infection. Later it was found that retroviral LTRs could also be used to enhance integration of Ads when included in the recombinant Ad [167,168]. In addition to retrovirus/Ad hybrids, AAV/Ad hybrids have been made. The packaging-capacity of AAV vectors is very limited compared to Ads. New strategies have emerged in which Ad sized AAV genomes were used in combination of the Ad packaging signal in the 5' AAV ITR. With these vectors, the large genome size of Ads is combined with the random integration capacity of AAV vectors. Even site-specific integration capacity is retained if Rep is present [169].

Sleeping Beauty. With the reactivation of the Tc1/mariner transposable element "Sleeping Beauty" Ads could be constructed containing a transposable element. In the presence of the transposase enzyme, this transposable element could jump from the vector into the genome of the host. The major advantage of this system is that a specific region of the Ad vector can be transferred without other viral sequences. Unlike Retrovirus or AAV vectors, where the complete recombinant viral genome is integrated, only relevant sequences could be transferred. A drawback of the sleeping beauty system is that the integration efficiency is inversely correlated to the transposon size. Also in the presence of transposase, the transposon can 'jump' from its initial integration site to other sites in the genome, increasing the risk of insertional mutagenesis.

φC31. The bacteriophage φC31 is capable to integrate AttP flanked DNA into the genome of its host, Streptomyces. Phage φC31 uses a relative conserved motive called, AttB in the host genome as preferred site of integration. The advantage over sleeping beauty is that this integrase has hardly any size restrictions. Furthermore, the integrase recognized some pseudo AttB sites in the human genome making it more or less site specific. The major disadvantage is that the integrase can only work on supercoiled circular DNA, making it harder to use this system in most conventional viral vector systems.

In this thesis we studied the bacteriophage Mu (figure 5) and Agrobacterium Tumefaciens integration systems (figure 6B). Because of their high integration efficiency and relative simplicity, they are potentially suitable mechanisms for integrating adenoviral vectors.

Bacteriophage Mu integration system

Bacteriophage Mu is capable of integrating 37 kb of viral DNA into the genome of *Escherichia coli*. The integration requires

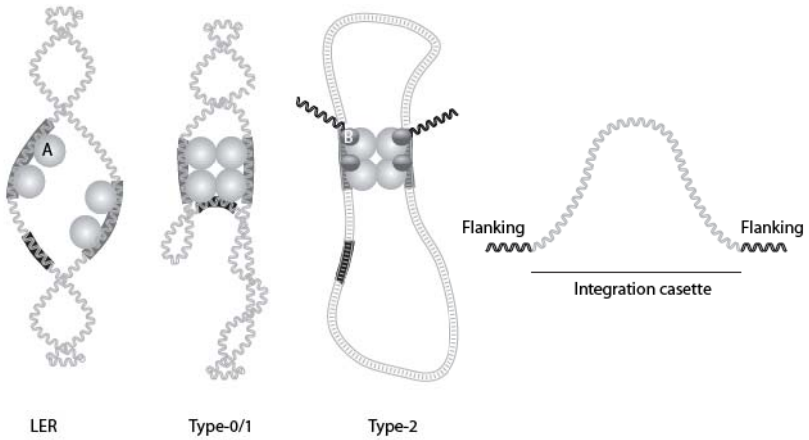

Figure 5. *Schematic representation of miniMu integration. The specific steps involved in Mu integration is described in the main text. The proteins MuA and MuB are marked with (A) and (B) respectively. The attL and attR sites are indicated by grey bars while the IAS is indicated by a black bar. Genomic sequences are indicated by the dark double helix.*

the phage to encode the 75 kDa MuA and 35 kDa MuB proteins, which can bind to two attachment sites located on the left and right genome termini (attL and attR). Monomers of MuA can bind these attachment sites sequence specifically [170,171]. The first complex that is formed is the LER, which consists of four MuA proteins bound to attL, attR and the internal activation sequence (IAS) as depicted in figure 5. The formation of this complex is enhanced by the *E.coli* protein Hu and metal ions [172]. When attL and attR are perfectly aligned the more stable type 0 complex is formed. The IAS helps in the correct positioning of the MuA subunits during the formation of the transposome. Without this IAS, transposition efficiencies drop approximately 100 fold [173]. The transition from the type-0 to the more stable type-1 complex is caused by MuA-induced nicking of both attL and attR. The binding of the MuA complex to both attachment sites is so strong that it prevents the formation of a relaxed formation of the plasmid even when the two single stranded breaks are introduced. Strand exchange with target DNA is finally induced by MuB [174-177]. Addition of MuB can increase the transposition 100 fold [178]. This type 2 complex is the most stable and requires ATP for the strand-exchange reaction.

Besides the phage-encoded MuA and MuB proteins, the integration also depends on host factors. For the actual transposition the *E.coli* genes *dnaE*, *dnaX* (subunits of the DNA Polymerase III holoenzyme), *dnaB* (major helicase at the replication fork), *dnaC* (promoted DnaB loading on the replication fork), *dnaG* (DNA primase), *gyrA*, and *gyrB* (two subunits of DNA gyrase) were found to be required for transposition to the host [179,180]. After integration, the very stable type-2 complex needs to dissociate from the host target sequence. The *E.coli* ClpX protein has been shown to be involved in the destabilization of this complex. Without ClpX, the recombination efficiency drops due to removal of the factor that changes the equilibrium between type-1 to type-2 transition. With the development of an *in vitro* miniMu integration system, only MuA in combination with the miniMu genome was shown to be essential [181,182].

The Agrobacterium tumefaciens integration system

Agrobacterium tumefaciens (*A.tum.*) has been a well-studied microbe especially since the discovery that it is the responsible agent

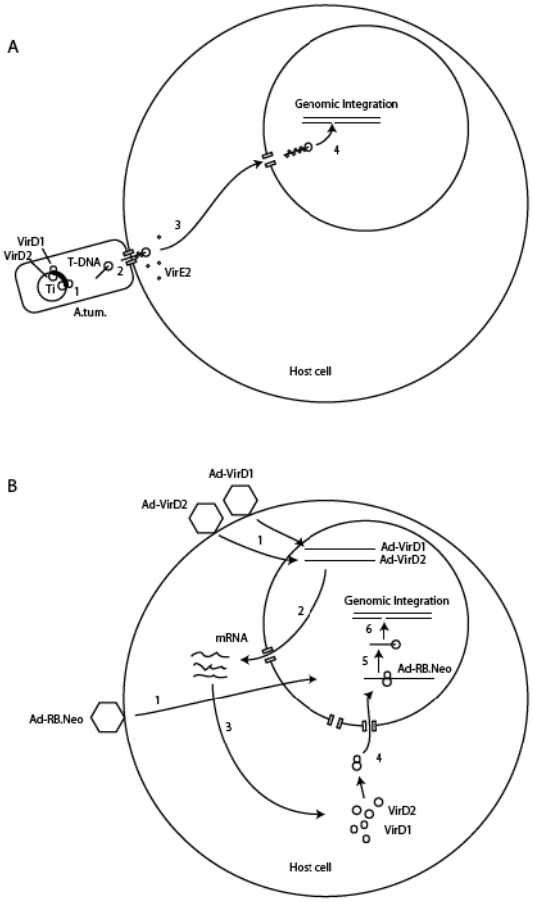

Figure 6. *Schematic presentation of the processes involved in A. tum. mediated T-DNA integration and the Adenivrus derived mechanism.*
A) A.tum. binds to the target cell. Phenolic compounds released by a wound activate expression of the Vir operon resulting in expression of VirD1 and VirD2 amongst others. VirD1/VirD2 complexes bind the left and right attachment sites (1) where VirD2 nicks and covalently binds the bottom strand. After strand displacement, VirD2 bound ssDNA T-DNA molecules are formed (2). These VirD2/T-DNA complexes are transported through a VirB channel which is formed between A.tum. and the target cell. The ssDNA binding proteins VirE2 is transported through this channel as well. In the plant cytosol, the T-DNA complex is coated by VirE2 which protects against degradation. The VirD2/VirE2/T-DNA complex translocates to the nucleus where it integrates in the host genome by illegitimate recombination involving host factors (4).
B)When a cell is infected with Ad's containg a VirD1 and VirD2 expression casette and an Ad containg the T-DNA (1), the cell will start to produce VirD1 and VirD2 proteins (2 and 3). The VirD1 proteins interact with VirD2 causing the VirD2 mediated tranlocation of the complec to the nucleus (4). The nuclear VirD1/VirD2 complex recognizes the Ad genome containg the T-DNA (5) and starts to produce VirD2/T-DNA complexes that integrate in the host genome by illegitimate recombination (6).

for causing crown-gall disease in plant stems (Smith and Townsend 1907). This crown-gall disease is recognized by the formation of large tumors on plant stems as a result of local uncontrolled cell division.

A.tum. is capable of injecting a single-stranded DNA (transferred DNA or T-DNA) molecule in dicotyledonous plant cells near a wound (see figure 6A for a schematic view). The *A.tum.* T-DNA synthesis machinery is activated by phenolic compounds released from the wound resulting in the activation of *A.tum.* proteins involved in T-DNA synthesis. Most of the factors involved in T-DNA

synthesis are located on the non-transferable part of the tumor-inducing plasmid. A special region of this tumor-inducing plasmid is the T-DNA, which can be recognized by two direct repeats (the left- and right border). Some of the genes located on this T-DNA encode for plant hormones promoting cell division. Others cause the production of opines, which is used as carbon-nitrogen source by *A.tum.* The left and right borders are recognized by a VirD1/VirD2 dimer, encoded by the *VirD* operon on the Ti-plasmid. VirD2 is able to cleave the bottom strand of the borders on single-stranded DNA sequence specifically. However, on supercoiled double-stranded DNA, VirD2 can only nick in the presence of VirD1. The function of VirD1 is to allow VirD2 to nick the single-stranded bottom strand on the Ti plasmid most likely by local unwinding of the double-stranded borders. After nicking, VirD2 is covalently bound to the 5' terminus protecting the DNA from exonucleases. The nicked strand is then removed from the Ti plasmid via a strand-displacement mechanism. The VirD2/T-DNA complex is subsequently transported to a membrane pore formed between *A.tum.* and the plant cell. This T-complex transporter (T-transporter) is formed by 11 proteins encoded by the *VirB* operon and VirD4. This T-transporter is able to transport the VirD2/T-DNA complex as well as individual proteins like VirE2, VirF and more recently VirE3 [183]. In the plant cytosol, the VirD2/T-DNA complex is bound by VirE2, protecting it from endonucleases. Both VirD2 and VirE2 contain nuclear localization signals causing the transport of the VirD2/T-DNA/VirE2 complex to migrate into the plant nucleus. Although both nuclear-localization signals are functional, only VirD2 seems essential for the delivery of T-DNA in the nucleus. The VirD2 protein was found to interact with cyclophilins [184], which are thought to play a role in protein folding and serve as molecular chaperones and transport intermediates. Cyclophilins are specific targets for many pathogens in the CsA-mediated attenuation of parasitic infections, including leishmaniasis, malaria and toxoplasmosis [185,186]. More recently, the VirD2-interacting protein GIB3, was isolated from a tomato cDNA library and it was shown to be able to block nuclear import of the T-DNA complex when overexpressed [187]. The nuclear localization of VirE2 is most effective in plant but less in other species. This species specificity was attributed to the binding of VirE2 to VIP1, which proved to be essential for nuclear localization of VirE2 [188]. A novel function of VirE2 was suggested by Dumas and colleagues, who showed that VirE2 could be incorporated into membranes and form voltage-gated, anion-selective, ssDNA-specific channels [189]. In the nucleus the T-DNA integrates in the host genome, an event that will eventually lead to the production of nopaline/auxin and opines.

Experiments performed by Ziemienowicz and colleagues demonstrated that for the *in vitro* ligation of T-DNA plant enzymes are required, but not VirD2 [190]. However, it is known that certain deletions in VirD2 can cause larger deletion in the integrated 5' end of the T-DNA, while in *wt* VirD2 the 5' end is relatively conserved after integration. This suggests a role for VirD2 during integration. To understand what host proteins are required for T-DNA integration, Attikum and colleagues used T-DNA transfer to several yeast strains having defects in the DNA repair machinery. With this system they were able to demonstrate that *Yku70* and *Rad52* are key regulators of T-DNA integration by directing T-DNA integration towards non-homologous recombination or homologous recombination, respectively [192,193]. If eventually the T-DNA integration process could be altered to making homologous recombination the preferred pathway, this would result in a system that could facilitate targeted transgenesis and gene repair, which is one of the standing goals in gene therapy.

Illegitimate recombination

While the mechanisms behind random integration are still not fully understood, some studies suggest that random integration is less random than previously thought. Rijkers and colleagues reviewed 35 transgenic mouse lines and found that some chromosomes were more often targeted for chromosomal integration than others [194]. At the locus level, it was found in 93% of the cases that illegitimate recombinations occured within 10 bp of a Topoisomerase I (Topo I) site. In addition to the Topo I sites, transgenes have been found near *E.coli* CHI elements and mini-satellite sequences [195-199]. These Topo I sites consist of AT and purine rich regions than can adopt a more open or bent DNA conformation. One putative mechanism is that these purine-rich regions are more susceptible for integration due to strand-invasion. One other mechanism is that these regions are more prone to double-strand breaks (DSB) and thus allow for integration via the DSB-repair machinery.

Analysis of T-DNA genome border sequences in 29,482 transgenic rice strains revealed that the integrations were randomly distributed among the chromosomes with slightly higher distributions at chromosome 1, 2, 3 and 6 [200]. Surprisingly, T-DNA was less prone to integration in known hot and cold spots compared to the Tos17 retrotransposable element. In contrast to the data described above, T-DNA rarely integrated in repetitive structures. A preference for T-DNA integration close to the start codon of known genes on chromosome 1 was observed. This suggests that T-DNA-mediated integration might be using a different repair mechanism although the DSB repair mechanism is also used [201,202]. In yeast, already three independent pathways have been identified. The majority of T-DNA integration occurs via homologous recombination dependent on Rad51 and Rad52. The main non-homologous recombination pathway is dependent on the non-homologous end-joining proteins Yku70, Rad50, Mre11, Xrs2, Lig4 and Sir4. A secondary minor telomeric integration pathway was found, which depended on Yke70 but not on Rad50, Mre11 or Xrs2 [192]. Furthermore, VirD2 has been shown to interact with TATA-box binding protein (TBP) in *Arabidopsis*. This interaction might link T-DNA integration to sites of active transcription and possibly to transcription-coupled repair (TCR) [191].

Disadvantages and risks of random integration

The major disadvantage of non-targeted vector integration in the host genome is the unpredictable effects on endogenous gene expression. When exogenous DNA is integrated in the host genome, chromosomal rearrangements can occur. Especially for the *A.tum.*-integration mechanism, many genomic rearrangements and deletions have been described in plants. It is not known whether these recombinations are a general effect of T-DNA integration, or result from the selection process that was performed to obtain cells that underwent T-DNA integrations.

Besides changes on sequence level, also the expression of surrounding genes might change. First, the integration of an exogenous cassette can lead to an altered methylation status of the integration locus leading to gene silencing or activation. Secondly, regulatory sequences in the exogenous expression cassette might affect expression of flanking genes.

Such an unfortunate example is the trial of 11 boys that suffer from X-linked Severe Combined Immune Deficiency (SCID) caused by a mutation in the common chain gamma (γc) protein which is a subunit of interleukin receptors 2, 4, 7, 9 and 15. Especially the interleukin-7 receptor is known to be important for the differentiation of T- and B-lymphocytes and Natural Killer cells. Due to the absence of a functional immune system,

these patients must recide in isolation tents to shield them from pathogens.
At very young ages, $CD34^+$ bone-marrow stem cells were isolated from these boys and infected with a replication-deficient Moloney Murine Leukemia Virus (Mo-MuLV) vector expressing the γc protein. After reimplanting the transduced bone-marrow stem cells, these boys started to develop normal numbers of CD4+ and CD8+ cells and responded well to childhood vaccinations including Polio, diphtheria and tetanus. Approximately four years after treatment an acute lymphoblastic leukemia was observed in one of the boys and two similar cases have followed since [203]. Analysis of the site of integration revealed that the Mo-MuLV vector was integrated in close proximity of the *LMO-2* gene, a known proto-oncogene. Since the chance that in two patients the retrovirus integrated at the same locus is extremely rare, either a prevalent site of integration was expected or the lymphomas were the result of some sort of selection procedure. Since preceding clinical trials where similar retroviral vectors containing different transgenes were used did not report the formation of leukemia's, the γc transgene was suspected. By searching a database containing 300 Mo-MuLV induced hematopoetic tumors, Dave and colleagues found 2 leukemias in which the LMO-2 locus was targeted and 2 leukemias in which the *IL2rg* locus (encoding the γc protein) was targeted. Of the latter one leukemia contained an integration at both *LMO-2* and *IL2rg* [204]. The authors argued that the probability of finding a leukemia with clonal integrations at both *LMO-2* and *IL2rg* by random chance is exceedingly small. This finding provided the evidence that both *LMO-2* and *IL2rg* could cooperatively act as oncogenes resulting in the formation of leukemia. More recent studies revealed that lentivirus expressing the *IL2rg* gene causes the formation of lymphomas which resulted in 33% of the cases in death of the animal within 81 days after treatment. Mice treated with cells that were mock transduced of infected with lentivirus expressing GFP were not affected [205]. This indicated that the oncogenic potential of LV-IL2rg is not the result of oncogenic elements in the lentiviral backbone or due to insertional mutagenesis per se.
The Mo-MuLV vector used in the trial did not contain a deletion in the promoter region of the LTRs (the so-called self-inactivating (sin) vectors). As a result the promoter activity in the 5' LTR and especially in the 3' LTR could enhance the transcription of genes located at the site of integration. The over-expression of the *LMO-2* gene could be potentially prevented by using sin vectors. Furthermore, promoter-trapping experiments showed that Mo-MuLV vectors do preferentially integrate near actively transcribed promoters while HIV vectors integrated throughout the transcriptional unit [206]. Though the later might increase the risk of insertional mutagenesis, it demonstrates that the choice of vector is important when establishing a therapeutic approach.

Taken together, in this thesis we aim at the improvement of the adenoviral vector system by achieving replication specificity and transgene integration. Replication specificity should increase the safety of the recombinant Ad vector even further while this technology might be used to produce gutless Ads free of helper contamination. Due to the absence of immunogenic helper Ads, these vector batches should yield prolonged transgene expression compared to treatments with first generation Ads. Integration of the transgene in the host genome should ensure transgene persistence while less virus is required when targetted to dividing tissues due to genome amplification. The use of a lower viral dose decreases the chance to trigger an immune responds. So, in this thesis we aim for Ad vectors with improved efficacy by adressing both replication specificity as well as transgene integration.

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CHAPTER 2

Efficient mobilization of E1-deleted adenovirus type-5 vectors by wild-type adenoviruses of other serotypes

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Efficient mobilization of E1-deleted adenovirus type-5 vectors by wild-type adenoviruses of other serotypes

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Mobilization of replication-deficient adenovirus vectors can lead to spread and shedding of the vector. Here we show that in cultured HepG2 cells wild-type adenoviruses of subgroup A (Ad12), B (Ad7, 11 and 16), C (Ad1, 2 and 5) and E (Ad4) can efficiently mobilize Ad5CMV*luc*, an Δ*E1*Δ*E3*-Ad5 vector carrying the firefly luciferase gene as reporter. In addition, we show that Ad5CMV*luc* can be propagated on Ad12-*E1*-transformed human embryonic retinoblasts. This provides evidence that expression of the *E1* region of Ad12 is sufficient for mobilizing Δ*E1*-Ad5-derived vectors. Thus, in therapeutic applications of replication-defective Ad vectors any active Ad infection is of potential concern, independent of the serotype involved. To prevent vector mobilization by *wt* Ads, new vectors should be developed in which essential functions such as the initiation of DNA replication and DNA the genome packaging are restricted.

Adenoviruses (Ad) are a diverse family of viruses. In humans at least 51 different serotypes have been described, which have been divided into 6 subgroups based on hemagglutination properties with red blood cells, oncogenic potential and G+C content of their DNA [1]. Replication-defective derivatives of serotypes 5 (Ad5) and 2 (Ad2), generated by deletion of sequences of the viral *E1*, *E2* and *E4* regions, have been widely used as gene-transfer vehicles in experimental gene therapy [2]. Therapeutic application of recombinant Ad vectors is complicated by the presence of replication-competent adenoviruses (RCA). The presence of RCA in the vector batches may lead to inflammation and tissue damage in the recipient [3], [4]. In addition, RCA can mobilize the Ad vector [5], and this could lead to increased vector shedding. The development and implementation of improved helper cells [6] and vectors [7], as well as stringent quality-control measures [8,3], can virtually eliminate the problem of RCA in the vector batches.

However, this does not solve the mobilization problem completely. It is conceivable that after receiving gene therapy, patients develop a viremia with a wild-type (wt) Ad. Indeed, administration of *wt* Ad5 into lungs of cotton rats previously exposed to replication-defective Ad5 vectors led to mobilization of the vector [5]. The risk of a mobilization by *wt* Ad of the same serotype is limited, as most patients will develop immunity against the vector serotype [9]. It is unknown, however, whether the vectors can be mobilized by other serotypes, viz. serotypes different from the one from which the vector had been derived. To study this possibility, we studied whether *wt* Ads of subgroup A, B, C and

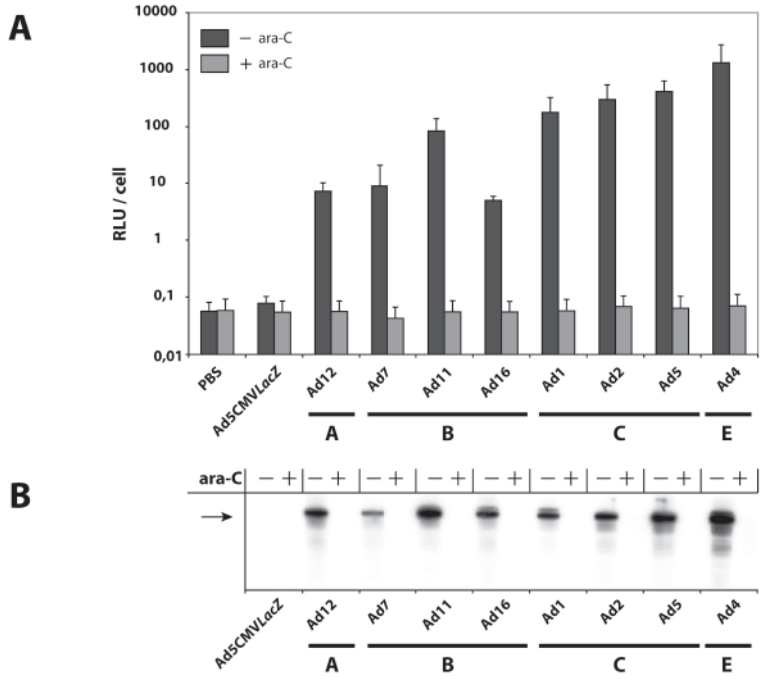

Figure 1. *Luciferase expression in HepG2 cells infected with mobilized Ad5CMVLuc. HepG2 cells were infected with Ad5CMVLuc (MOI = 10) and 24 hours post-infection, co-infected with wt Ads (MOI = 5) in the presence or absence of 20 µg/ml ara-C. As negative controls, Ad5CMVluc-infected cells were co-infected with Ad5CMVLacZ (MOI 5) or mock-infected, either with or without ara-C. Cells were harvested forty-eight hours post co-infection and freeze-thawed three times to release the virus. Dilutions of the virus were used to infect HepG2 cells. Eighteen hours after infection cells were lysed and assayed for luciferase activity (**A**). The graph depicts the means (±1 S.D.) of three experiments. In parallel, low-molecular-weight DNA was extracted from infected cells according to the Hirt method. DNA was digested with PvuI. Ad5CMVLuc DNA was detected by a radiolabeled Luc-specific probe in Southern analysis (**B**). The arrow indicates the hybridizing 6-kb luciferase-gene fragment.*

E could mobilize the vector Ad5CMV*luc*, an Δ*E1*Δ*E3*-Ad5 vector carrying the firefly luciferase gene as a reporter.

Mobilization of Ad5 vectors by different serotypes

To assess whether *wt* Ads can mobilize a Δ*E1*Δ*E3*-Ad5-derived vector we set up a marker- mobilization assay. HepG2 human hepatoma cells were cultured in 24-well plates in DMEM containing 8% FCS. At 90 % confluency the cells were infected with a RCA-free stock of Ad5CMV*luc* at a multiplicity of infection (MOI) of 10. Twenty-four hours post-infection the cells were washed with PBS and exposed, at a MOI of 5, to stocks of Ads of subgroups A (Ad12), B (Ad7, 11 and 16), C (Ad1, 2 and 5) and E (Ad4) that had been purified twice on CsCl gradients. All adenovirus titers were determined by plaque assay on 911 cells. At an MOI all *wt* Ads gave complete CPE at 60 to 120 hours post infection (data not shown). As controls, HepG2 cell cultures were infected with the Δ*E1*Δ*E3* deleted vector Ad5CMV*lacZ*, or mock infected. In a parallel experiment, the assay was performed in the presence of 20 µg/ml cytosine-arabinoside (ara-C) in the culture medium. This concentration of ara-C effectively inhibits Ad replication in HepG2 cells (data not shown). Three days after the initial infection, the cytopathogenic effect was apparent in all cultures. At that time,

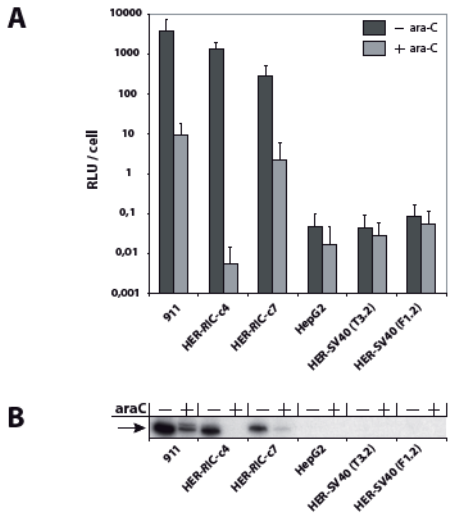

Figure 2. *Mobilization of rAd5Luc by trans-complementation of Ad12 E1. HepG2, 911, HER-RIC-c4, HER-RIC-c7, HER-SV40(T3.2) and HER-SV40(F1.2) cells were exposed to Ad5CMVluc in the presence or absence of ara-C. Forty-eight hours post infection, cells were harvested and the virus was released by three cycles of freeze-thawing. Subsequently, HepG2 cells were exposed to dilutions of the sample to quantify the amount of luciferase virus. Eighteen hours post infection, cells were lysed and assayed for luciferase expression (**A**). The graph depicts the means (±1 S.D.) of three experiments. In parallel, low-molecular-weight DNA was extracted from these cells according to the HIRT method. DNA was digested with PvuI. Ad5CMVLuc DNA was detected by a radio labeled Luc fragment in Southern analysis (**B**). The arrow indicates the hybridizing 6 kb luciferase-gene fragment.*

the cells were harvested with the medium and the virus was released from the cells by three cycles of freeze-thawing. After the last cycle, the lysates were cleared by centrifugation for 2 minutes at 1600 *g*. The amount of mobilized Ad5CMV*luc* could be determined by measuring the amount of luciferase virus present in the lysate. To quantify the luciferase virus, HepG2 cells were infected with adenovirus derived from the HepG2 lysates and luciferase activity was determined 18 hours post infection. To inhibit replication of the *wt* Ads present in the lysates, which may affect the luciferase activity, 20 µg/ml ara-C was added to the medium of the indicator cells. The results of the mobilization assays are depicted in figure 1A. Luciferase expression in HepG2 cells infected with virus derived from the Ad5CMV*luc* and Ad5CMV*lacZ* coinfected cells was as low as in HepG2 cells infected with virus derived from Ad5CMV*luc* and PBS treated cells. So, neither in the mock-infected cells, nor in the Ad5CMV*lacZ* infected cells any significant mobilization had occurred. In contrast, luciferase activity was readily detectable in all assays where *wt* Ads had been added, suggesting that all serotypes could mobilize Ad5CMV*luc*. Similar results were obtained when the cells were harvested 5 days after infection with Ad5CMV*luc* (data not shown). The amount of Ad5CMV*luc* produced upon coinfection by *wt* Ad5 was only 10-fold lower than the yield of Ad5CMV*luc* in the E1-complementing cell line 911 (compare the luciferase activity per cell in the wtAd5 co-infected cells in figure 1A with the 911 levels in figure 2A).

To confirm replication of Ad5CMV*luc* DNA after infection of the cells with *wt* Ads, low-molecular weight DNA was extracted by Hirt isolation [10] from HepG2/Ad5CMV*luc* cells infected with the various wt Ads. Southern analysis of *Pvu*I-digested Hirt DNA with a luciferase- gene-specific probe demonstrated replication of the Ad5CMV*luc* vector (fig. 1B). The replication (fig. 1B), as well as the mobilization assays (fig. 1A), were sensitive to 20 µg/ml ara-C, indicating that replication is essential for mobilization of the vector. The data suggest that all Ads serotypes tested can mobilize the Ad5CMV*luc* vector.

Mechanisms of complementation

Two mechanisms could contribute to the replication and mobilization of the Ad5CMV*luc* vector. Firstly, the *E1* region of the *wt* virus could functionally complement the *E1* deletion of the vector, and activate expression of the Ad5 genes of the vector. In

the '*E1* complementation' model the vector would be replicated by and packaged in its homologous proteins. Thus, the presence of the E1 proteins of the *wt* Ad would be sufficient to mobilize the vector. Alternatively, in the 'facilitated replication & packaging' model, the *wt* Ad's *E1* region alone is unable to activate expression of the vector genome. Hence, mobilization can only occur if the *wt* Ad-derived proteins other than or in addition to E1, facilitate replication and packaging of the vector DNA.

Mobilization by E1 complementation

To test whether expression of E1 is sufficient to replicate the Ad5CMV*luc* vector we employed a panel of human embryonic retinoblasts (HER) cell lines transformed with the *E1* region of Ad12, and as controls, cells transformed with the SV40 early region [11]. As negative and positive controls we used cell lines HepG2, and 911 [12], a HER line transformed with the *E1* region of Ad5, respectively. These cell lines were infected with the Ad5CMV*luc* at a MOI of 5. At 3 days post infection, the cells and media were harvested, freeze-thawed (3 cycles), and assayed for the presence of the Ad5CMV*luc* vector by infecting HepG2 cells with adenovirus derived from the HepG2 lysates followed by luciferase assay (fig. 2A). Ad5CMV*luc* propagation was detectable neither in the HepG2 cells, nor in the SV40 transformed HER-SV40(T3.2) and HER-SV40(F1.2) cells. In contrast, the HER-RIC-c4 and HER-RIC-c7 cells produced readily detectable amounts of reporter virus. The amplification factor, that is the ratio of the Ad5CMV*luc* production in the cultures divided by the Ad5CMV*luc* production in the araC-treated cultures, was only moderately lower than the amplification factor obtained in the Ad5*E1*-transformed 911 cells. Virus replication was sensitive to Ara-C. Similar results were obtained if the viruses were harvested 5 days post-infection (data not shown). Southern analysis confirmed replication of the vector in the Ad5*E1*- and Ad12*E1*-transformed HER lines, and not in the SV40-transformed HER lines (fig 2B). Taken together, these data show that *wt* Ads of subgroups A, B, C and E can mobilize Δ*E1*Δ*E3*-Ad5 vectors and that for Ad12 at least, *trans*-complementation by E1 is sufficient for mobilization at least for Ad12 E1.

Our data confirm and extend the earlier observation that *Bam*HI digested *wt* Ad12 (subgroup A) and Ad7 (subgroup B) could efficiently complement an *E1* deletion of Ad2 (subgroup C) [13]. Also hybrid Ad5 and Ad12 viruses in which the *E1* region had been replaced (in part) by the corresponding region of Ad12 and Ad5, respectively, demonstrated to be viable [14]. However, another study showed that a defect in the *E1* region of Ad2 could only be complemented poorly by Ad9 (subgroup D) [15]. In addition, we show here that Ad12*E1*-transformed HER cell lines complement the *E1* deletion in Ad5 and allow the Ad5 vector to be propagated.

It remains to be established if the 'facilitated replication & packaging' model really contributes to the mobilization of Δ*E1*Ad5 vectors. It has been shown that Ad5-derived helper-dependent Ad vectors can be amplified and packaged into Ad2-derived capsids [16], demonstrating the feasibility of the model. It should be borne in mind, however, that that Ad2 and Ad5 are highly similar 'twin' serotypes and have origins of DNA replication and packaging signals that are identical in sequence. Indeed it has been shown recently that Ad12 (subgroup A), Ad7 (subgroup B) and Ad 17 (subgroup D), cannot complement the defect of Ad5 mutant *pm8001* [17]. This mutant cannot package its DNA due to a mutation in the L1 52/55kDa gene. This indicates that the DNA packaging system of different serotypes cannot productively interact with Ad5 DNA. Thus mobilization by E1 complementation appears to be the more general mechanism.

Our data demonstrate of mobilization of Ad5-derived vectors can be provoked not only by *wt* viruses of the homologous serotype, but also by different serotypes. Thus, in therapeutic applications of replication-defective Ad vectors any active Ad infection is of potential concern, independent of the serotype involved. To prevent vector mobilization by wt Ads, new vectors should be developed in which essential functions such as the initiation of DNA replication and DNA the genome packaging [5] are further restricted.

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CHAPTER 3

Relaxed Template Specificity In Fowl Adenovirus-1 DNA Replication Initiation

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Relaxed Template Specificity In Fowl Adenovirus-1 DNA Replication Initiation

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The Fowl Adenovirus 1 (FAdV-1) isolates PHELPS and OTE are highly homologous but have striking differences in the repeat region of the terminal repeat (ITR). While the repeat region in OTE conforms to the conventional human adenovirus repeat region (5'-CATCATC), PHELPS contains guanidine residues at position 1,4 and 7 (5'-GATGATG). Therefore, the FAdV-1 is currently the only known species, which contains Ads starting with guanidine residues. This implies that the FAdV-1 isolates PHELPS and OTE either have distinct template specificity at replication initiation, or alternatively, they have a relaxed specificity for replication initiation. In this study we confirmed the distinct sequence variation at the origin of DNA replication in the ITR of the FAdV-1 PHELPS and OTE isolates. Sequence analyses of the *pTP* and *Pol* genes of both PHELPS and OTE did not reveal differences that could explain the distinct template specificity. Replication assays demonstrated that linear DNA fragments flanked by either 5'-CATCATC or 5'-GATGATG termini replicated in cells upon infection with FAdV-1 OTE and FAdV-1 PHELPS. This was evident from the appearance of *Dpn*I-resistant fragments in a mini-replicon assay. From these data we conclude that the FAdV-1 has relaxed, rather than changed, its template specificity at replication initiation.

Adenoviruses (Ads) are linear double-stranded DNA viruses with genomes 34 to 48 kb in size and with terminal proteins (TP) covalently bound to the 5'ends [1-3]. At the ends an inverted terminal repeat (ITR) is located, containing the origin of replication [4-16]. The Ad genome is replicated from each end of the molecule via a strand-displacement mechanism. Most of the insight in the molecular mechanism of Ad DNA replication is derived from studies of the HAdV-5 and HAdV-2 (species HAdV-C). Preceding the initiation of replication, two viral proteins, the precursor of the terminal protein (pTP) and the Ad DNA Pol, form a stable heterodimer [17,18]. Although this complex can initiate DNA replication, the initiation is strongly stimulated by the host proteins Oct-1 [19,20] and NF-I [21]. The pre-initiation complex of pTP/Pol/Oct-1/NF-I bound to the origin initiates replication with a preference for the nucleotide at position 4 in the template strand. A specific amino acid in pTP, Ser^{580}, is used a primer for covalent binding of dCMP by

pol, generating pTP-C [22,23]. After formation of a pTP-CAT trinucleotide complex, the complex jumps back to allow base-pairing of the CAT with the first three nucleotides of the template strand [24]. After dissociation of the pTP/Pol complex the elongation can proceed requiring a third virus encoded protein, the DNA-binding protein (DBP), involved in unwinding the dsDNA ahead of the polymerase [25]. The pTP remains bound at the 5' end of the genome and protects against degradation by exonucleases. Later during infection, pTP is cleaved by Ad protease to form the Terminal Protein (TP) [26]. The jumping-back mechanism explains the short 3 (infrequently 2 or 4) base-pair direct repeat found at the termini of all Ad ITRs.

The first step of DNA replication is covalent coupling of dCMP to the pre-initiation complex. This step is well conserved. All Mastadenoviruses and Atadenoviruses characterized to date have a C residue at the 5' end of their genome, suggesting a preference of pTP to bind dCMP. This concept is supported by work of King and van der Vliet [24] who showed that mutation of G4 in the template strand blocked replication initiation of HAdV-5 in-vitro. Even in the absence of any template DNA, pTP-C/Pol complexes were formed [24]. The preference of pTP to bind dCMP was reduced in the presence of manganese ions in the replication initiation assay, suggesting that structural factors govern the template specificity.

Strikingly, whereas most of the Aviadenoviruses conform to the C rule there is an exception in the Fowl adenoviruses 1 (FAdV-1). The PHELPS isolate (gi:1314432) of the Chicken Embryo Lethal Orphan (CELO) diseases-causing Ad and isolate KUR (gi:209935) are unique among Ads in that their genomes start with the sequence 5'-GATGATG. Alignment of the KUR sequence showed that it was 99% identical to PHELPS within the first 168 bp of the right ITR. Intriguingly, another FAdV-1 isolate, OTE (gi:210033, gi:210032), of which only the sequence of the first 68 bp of the ITR has been published [27], is distinct from PHELPS and KUR in that it conforms the Ad convention and starts with the sequence 5'-CATCATC.

The genome sequence of the PHELPS isolate has been completely determined by Chiocca *et al.* [28]. The virus lacks homology with the *E1*, *E3* and *E4* regions of the Mastadenoviruses as well as the genes encoding pV and pIX. The *E2* and Late gene clusters of PHELPS are homologous to the Mastadenoviruses. Open reading frames (ORFs) homologous to HAdV-5 DBP, pTP and Pol could be identified in PHELPS (gi:1314452, gi:1314443 and gi:1314442, respectively). The 54 bp ITRs are relatively short in PHELPS compared to the 103 bp found in HAdV-5.

The unconventional occurrence of G nucleotides at position 1, 4, and 7 of some FAdV-1 isolates, as well as marked heterogeneity between different FAdV-1 isolates is intriguing. Here we confirmed the sequence difference between the FAdV-1 PHELPS and OTE and characterized their

Table 1. *Synthetic oligonucleotides used in this study.*

CELO-PE	5'-GCGTCAGTATTGGTTAGTTTTGAGG
MiniCeloL-AS	5'-GAGGGCGTCGCGAAGATCTCGTCTAAGAGGAAATACAAGAAAAAACAG
oligo-dT-20	5'-TTTTTTTTTTTTTTTTTTTT
RTCptp-rev	5'-GTGGGCTATCGTAACCTATCTGCA
RTCpol-rev	5'-*CGG*TATTTTGTGAA*CGGAGCC*
CeloRTptp-for1	5'-AGGAGGAATCGACGTCGGCG
CpTP-as	5'-TAAAGCTTGCCACCATGGCGGGGACGGGGTGCATTACG
CtPT-s	5'-TGAATTCTTACAGAGGCTGACCTCGTCG
CELO-pol-s	5'-TGACGCGCTGGTAGAAGTCCCGCGAG
CELO-pol-as	5'-CCATCGCTACCAACGAGGAGATCAAAGCCAAC
CELO-pol-F4	5'-GTCTAGCCAATAGAAGCCTTCGTC
CELO-pol-R5	5'-CCTCAGACGCGTGCTCCG
BamITR-C	5'-CGCGGATCCCATCATCTATAATAACCTCAAAAACTAACGCAG
BamITR-G	5'-CGCGGATCCGATGATGTATAATAACCTCAAAAACTAACGCAG
MiniCeloL-AS	5'-GAGGGCGTCGCGAAGATCTCGTCTAAGAGGAAATACAAGAAAAAACAG
MiniCeloR-S	5'-TTAGACGAGATCTTCGCGACGCCCTCTATAGACATTATATAGAATATAACTG
cGFP-for	5'-GGGGTCATTAGTTCATAGCCCAT
cGFP-rev	5'-GCTTGATCCAAATCCAAACAGAGT

pTP and Pol genes. In addition we provide evidence that these viruses have a relaxed, rather than a changed, template specificity.

Materials and methods

Tissue culture and virus propagation. The PHELPS virus was obtained from the ATCC (VR-432, ATCC, Manassas, VA) and OTE was kindly provided by Dr. Matt Cotten (IMP, Vienna, Austria). Both PHELPS and OTE were propagated on the chicken hepatocellular carcinoma epithelial cell line, LMH (CRL-2117, ATCC). LMH cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 8% fetal calf serum (FCS), antibiotics and 3 g/l glucose in a 5% CO_2 atmosphere at 37°C. Dilutions of PHELPS or OTE were added to subconfluent LMH cells in DMEM supplemented with 2% Horse Serum (HS), antibiotics and 3 g/l glucose. Two hours post infection, the inoculum was replaced with DMEM supplemented with 8% FCS, antibiotics and 3 g/l glucose. When almost all cells showed cytopathogenic effect (CPE), approximately three days post infection, the cells were harvested in phosphate buffered saline (PBS) supplemented with 2% HS, and freeze/thawed to release virus particles. These freeze/thaw lysates were used for re-infections and mini-replicon assays.

ITR sequence determination. Virus particles from the freeze/thaw lysates were purified by CsCl density gradient as described [29]. Isolated particles were incubated with Proteinase K in 0.2% SDS, 8 mM EDTA, and viral DNA was extracted by phenol/chloroform and ethanol precipitation.
Primer extension assay: The primer CELO-PE was radiolabelled using T4 polynucleotide kinase and γ^{32}-ATP and elongated with T7 DNA polymerase in the presence of dATP, dTTP and either dCTP or dGTP. Elongated primers were size fractionated by electrophoresis on an 8% poly-acrylamide gel. For detection of the radio labelled fragments, a Kodak XAR film was used (Kodak, Vianen, The Netherlands).
Terminal transferase: A poly-A tail was added to the 3'end of the viral termini using terminal transferase (Promega, Leiden, The Netherlands). The first 800 bp of the left ITR were amplified by PCR using the primers MiniCeloL-AS and oligo-dT-20 (see Table 1). The PCR fragments were cloned in pCR2.1+ using the TA cloning kit (Invitrogen, Breda, The Netherlands) and used for sequence analysis.

Splice-site determination by rtPCR. LMH cells were infected with OTE and 18 hours post infection mRNA was isolated with the RT-PCR miniprep kit (Stratagene Europe, Amsterdam, The Netherlands). For the synthesis of cDNA, 1 µg mRNA was used in a reverse-transcriptase reaction (Promega) using the RTCptp-rev and RTCpol-rev primers to synthesize pTP and Pol cDNA respectively. For amplification of the spliced pTP and Pol fragments, the forward primer CeloRTptp-for1 was used in combination with RTCptp-rev or RTCpol-rev (see Table 1) to synthesize the pTP or Pol spliced fragments respectively in a standard PCR reaction using Taq polymerase (Perkin-Elmer Life Science, Hoofddorp, The Netherlands). The pTP and Pol PCR products were cloned in a pCR2.1 vector, and sequenced.

Isolation and sequencing of PHELPS and OTE DNA. For the isolation of pTP and Pol genes, viral DNA was isolated as described above. The pTP genes were isolated using the primers CpTP-as and CtPT-s (see table 1) in a standard PCR reaction. The 2.0 kb PCR product was digested with *Hind*III and *Eco*RI (MBI Fermentas GmbH, St. Leon-Rot, Germany) and cloned in a 5.4 kb *Hind*III/*Eco*RI fragment of pCDNA3.1+ and sequenced. Differences with the published PHELPS pTP sequenced were confirmed by direct sequencing of viral DNA.
For OTE pol, a 3.9 kb PCR fragment was amplified using the primers CELO-pol-s and CELO-pol-as and cloned in a 2.7 kb *Eco*RV digested pIC20H vector. This vector was used to sequence the OTE Pol gene.
Mini-replicon assay. The left and right genome ends of FAdV-1 OTE PCR amplified with the primers BamITR-C or BamITR-G together with MiniCeloL-AS for the left end and MiniCeloR-S for the right end. The left and right genome ends of FAdV-1 PHELPS PCR amplified with the primers BamITR-G together with MiniCeloL-AS for the left end and MiniCeloR-S for the right end (see Table 1; *Bam*HI sites are underlined in the primer sequence). By virtue of the homology in the Left ITR fragment and Right ITR fragments, both products could be linked in a second PCR reaction using either BamITR-C or BamITR-G primers and *Pfu* polymerase (Stratagene Europe). The resulting 2 kb PCR fragment was cloned in a pCR2.1+ vector using the TA-cloning kit (Invitrogen). The resulting plasmids, miniOTE-C, miniOTE-G and miniPHELPS-G were digested with NruI and ligated with a 2KB CMV-GFP cassette. The CMV-GFP cassette was synthesized with PCR from pShuttle-GFP using the primers cGFP-for and cGFP-rev and *Pfu* polymerase. The GFP cassette is used as a marker for transfection efficiency.
Subconfluent cultures of LMH cells were infected with OTE or PHELPS (m.o.i. = 1) in DMEM supplemented with 2% HS, antibiotics and 3 g/l glucose in a 5% CO_2 atmosphere at 37°C. Two hours post infection, inoculum was replaced with DMEM supplemented with 8%FCS, antibiotics and 3 g/l glucose in a 5% CO_2 atmosphere at 37°C. Six hours post infection, cells were transfected with 1 µg *Bam*HI digested miniOTE-C, miniOTE-G or miniPHELPS-G replicon plasmid, with the aid of JetPEI (Polyplus-transfection, Illkirch Cedex, France). Transfection efficiencies of 40% to 50% were achieved routinely. Viral DNA and mini-replicon DNA was isolated 54 hours post infection from LMH cells using HIRT small molecular DNA isolation procedure [30]. From the isolated DNA, 10 µg aliquots were digested

with either *Dpn*I, *Mbo*I or left undigested, and loaded on a 1% agarose gel. After electrophoresis and blotting the mini-replicon fragments were detected by Southern analysis using a radiolabelled GFP fragment as probe. For the detection of the mini-replicon backbone, a radioactive Amp-specific probe was used. It was noted that the presence of Adeno-Associated Virus in the assay severely reduced the efficiency of mini-replicon replication.

Sequnces submitted to GenBank. The coding sequences *pTP* and *Pol* genes of the FAdV-1 OTE isolate are deposited as AY421748 and AY421749, respectively. The genomic sequences of the *pTP* and *Pol* coding regions of the OTE isolate are deposited as AY421752 and AY421753. The first 773 nt of the left genome end and 819 nt of the right genome end of the OTE isolate are deposited as AY421750 and AY421751, respectively.

Results

The linear genomes of all Ads characterized to date start with a small direct repeat of 2, 3 or 4 nucleotides (Fig. 1A). To study the apparent diversity of the terminal sequences, PHELPS and OTE were propagated on LMH cells. To verify the nucleotide sequences of the PHELPS and OTE termini, the left end of their genomes were PCR amplified with DNA isolated from both PHELPS and OTE infected LMH cells as templates. The 3'-end of the bottom strand of the left-hand terminus was extended with an oligo-dA tail with the aid of terminal transferase. PCR amplification of the ITR sequences was performed using an oligo-dT primer and the FAdV-1-specific primer MiniCeloL-AS. The PCR fragments were cloned, and the plasmid clones were used for sequence analyses (accessions AY421750 and AY421751 for the left and right genome ends). The results confirmed the PHELPS and OTE terminal sequences to read 5'-GATGATG and 5'-CATCATC, respectively (Fig 1B). The homogeneity of the PHELPS and OTE terminal sequences was confirmed by primer extension assays directly on the isolated virus DNA (Fig 2). This assay determines the nucleotide at position 7 in the template strand of the ITRs. Primer extension on PHELPS DNA yielded a 34-nt product when dCTP was

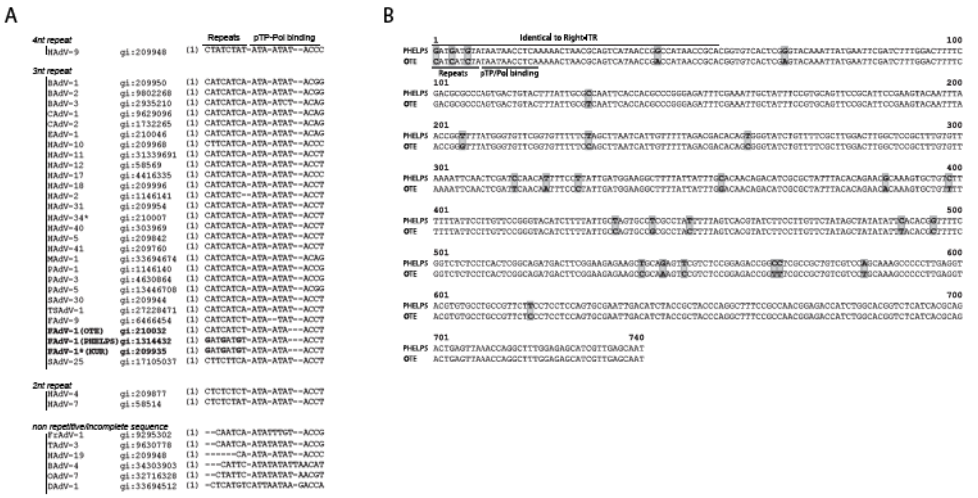

Figure 1. *Sequence conservation of the Adenovirus ITRs. (A) BLAST alignment of published Ad ITR sequences. The repeat region, pTP/Pol binding site, and the size of the repeats are annotated. The nucleotide differences in PHELPS and KUR are depicted in bold. (B=Bovine, C=Canine, D=Duck, E=Equine, F=Fowl, Fr=Frog, H=Human, M=Murine, O=Ovine, P=Porcine, S=Simian and TS=TreeSrew (Tupaia)). (*) The HAdV-34 and FAdV-1 KUR sequences are derived from the right ITR. (B) Differences between PHELPS and OTE in the first 740 nt of the Left-ITR. Two independent PCR isolates of the left ITR of OTE were sequenced and aligned against PHELPS. The repeat region and pTP/Pol-binding domain, as determined for HAdV-5, are indicated as well as the region identical to the right-ITR of PHELPS.*

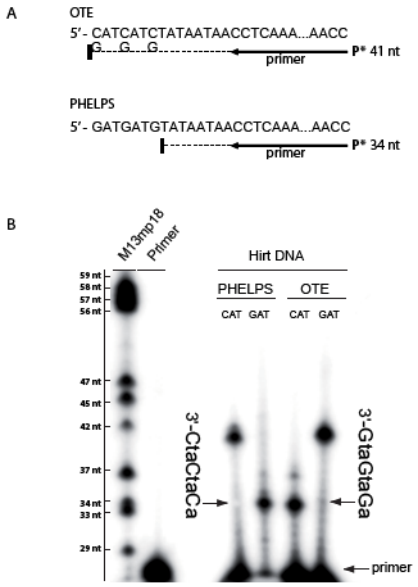

Figure 2. *Identification of nucleotide 7 in the left ITR of PHELPS and OTE. Genomic DNA was extracted from CsCl purified viral particles. A radioactive labelled probe was elongated with nucleotide mixtures depleted for dGTP or dCTP. (A) Without dCTP in the elongation mix, PHELPS would yield a product of 41 nt and OTE would yield a 34 nt product due to template dependent requirement of dCTP incorporation. (B) Elongated samples were denaturated and loaded on a sequencing gel. As a marker the ddGTP sequence lane of phage vector pM13mp18 is included.*

omitted from the elongation mix, while OTE yielded a 41-nt fragment. Primer extension in the presence of dCTP, but in the absence of dGTP generated a 41-nt fragment in PHELPS, but a 34-nt product in OTE. These data confirm the presence of guanine for PHELPS and cytosine for OTE at position 7. Furthermore, the virtual absence of the 41-nt elongation products in the lanes containing the 34-nt product confirms the homogeneity the terminal sequences in both isolates.

To study whether the difference in the initiating nucleotides is correlated with changes in the *pTP* and *Pol* sequences, the large exons containing the majority of the open reading frames for pTP and Pol from OTE DNA were cloned and sequenced. The genes were cloned by PCR from genomic OTE and PHELPS DNA and sequenced (accessions AY421752 and AY421753 for the genomic sequences and AY421748 and AY421749 for *pTP* and *Pol* cDNAs, respectively). Differences between our PHELPS sequence and the published sequence, and differences between OTE and PHELPS, were confirmed by direct sequence analyses on genomic DNA.

In PHELPS, open reading frames have been annotated for pTP (E2B pTP, AAC54905.1) and polymerase (E2B Pol, AAC54904.1). However, in HAdV-5 and HAdV-2, the translation of pTP and Pol messages are complex. The pTP and Pol proteins are translated from distinct messengers but use the same translation start codon. The majority of the coding sequences from pTP and from Pol are located in large distinct exons, but the translation initiating ATG is derived from a common upstream exon. The sequences preceding the first ATG of the large exons are essential for the activity of both proteins [31,32].

To determine the structure of the Pol and pTP mRNAs, rtPCR on RNA isolated from OTE infected LMH cells was used to characterize the presence of the upstream exon homologous to the human Ads. For cDNA synthesis we used primers RTCpol-rev for Pol and RTCptp-rev for pTP, both located downstream of the first ATG in the large exon. The forward primer (CeloRTptp-for1) was used for both OTE pTP and Pol mRNA's, and was based on sequence similarity in the shared 5' untranslated region (UTR) of HAdV-5 pTP/Pol mRNA and PHELPS genomic DNA (figure 3). The PCR products were cloned and the resulting plasmid clones were sequenced. Splice sites were predicted by aligning the cDNA fragments against the genomic sequence of PHELPS using the Sim4 algorithm [33]. Similar to HAdV-5, OTE pTP and Pol use a splice-donor site, 3 nucleotides downstream of an ATG sequence (nt 15081 in the published PHELPS sequence). The splice-acceptor site for pTP is located 159 nt upstream of the annotated ORF (nt 12155 in

published PHELPS sequence). The resulting mRNA would encode a 630 amino-acids pTP protein. This is 55 amino-acids larger than PHELPS pTP encoded by the annotated pTP ORF (AAC54905.1) and contains a conserved sequence motif [A]-[RHD]-[L]-[T]-[GN]-[Q]. The splice-acceptor site of Pol is located 610 bp upstream of the ORF (identical to nt 10476 in PHELPS) inside the pTP ORF (Figure 3). However, unlike HAdV-5, translation starting from the ATG, 6 nucleotides upstream of the splice donor, does not result in a *bona fide* Pol protein, whereas OTE and PHELPS Pol use the same splice donor, an alternative ATG must be used for the translation of Pol.

The first ATG downstream of the splice site is located at nt 10268 in both PHELPS and OTE and is in frame with the identified ORF. Translation from this start codon results in a 1255 aa polymerase protein, 134 aa larger than the annotated ORF in PHELPS (AAC54904.1). Both the pTP and Pol start codons confirm the minimal Kozak sequence (taaATGG and GaaATGG respectively). An overview of the organization is provided in figure 3.

Having established the putative ORFs made it possible to compare the sequence of the OTE pTP and Pol with the published sequence of PHELPS and other Ads (Figure 4A). We noted two differences between our sequence of PHELPS pTP and the published sequence. Our PHELPS contained $Arg^{325}Asn^{326}$ rather than Cys^{325} and Ser^{326} (numbered aa^{270} and aa^{271} in AAC54905.1). OTE and FAdV-10 are identical to our PHELPS sequence in this region. Only two differences were found between the pTP ORFs of PHELPS and OTE. PHELPS Val^{50} and Asp^{604} were changed in Leu and Glu respectively in OTE. These changes do not result in an altered amino-acids charge. In addition, the Val^{50} to Leu maps in the N-terminal part of the protein, which is unlikely to be involved in priming. Taken together these data suggest that the difference of initiating nucleotide is not caused by a functional change in terminal protein activity.

At the initiation of DNA replication Pol catalyses covalent binding of the first dCMP nucleotide to Ser^{580} of pTP. To exclude that the variation in initiating nucleotide is the result of a change in Pol function, we compared the OTE Pol sequence with the published PHELPS sequence. Eight base alterations were detected that would change the amino-acid sequence. The His^{416} to Gln and His^{455} to Gln (PHELPS; AAC54904.1 compared to OTE) alterations map in the exonuclease region of the polymerase and are therefore unlikely to be associated with the difference in initiation template-specificity. The amino acid changes Pro^{587} to His and Gly^{885} to Glu do not map in conserved regions of Pol. The changes Glu^{672} to Gly, Asn^{854} to Ser, Ser^{965} to Asn and Thr^{1203} to Ala, all map near the active site of Pol, but only the Ser^{965} to Asn is located in a conserved region. The localized sequence-similarity alignment algorithm (MACAW [34-36]) was used to find homologous-sequence blocks in the HAdV-5 and PHELPS Pol sequences. The amino-acid changes between the PHELPS and OTE sequence were compared with the HAdV-5 map and with BLAST alignments of several Ad polymerases (Fig. 4B).

The sequence comparisons of the *pTP* and *Pol* genes of PHELPS and OTE did not reveal obvious differences that would explain the difference in template specificity. Therefore it is tempting to speculate that the distinct differences at the origin of replication are not caused by a change of template specificity inherent to the pTP and Pol, but rather that the FAdV-1 Ads have a relaxed specificity. This would allow replication initiation on different templates. As a result, the 5-'GATGATG and 5'-CATCATC of PHELPS and OTE would be simply maintained by their virtue of the availability of either one of their templates sequences, and not by specificity of the pTP and/or Pol.

To test this hypothesis we constructed mini-

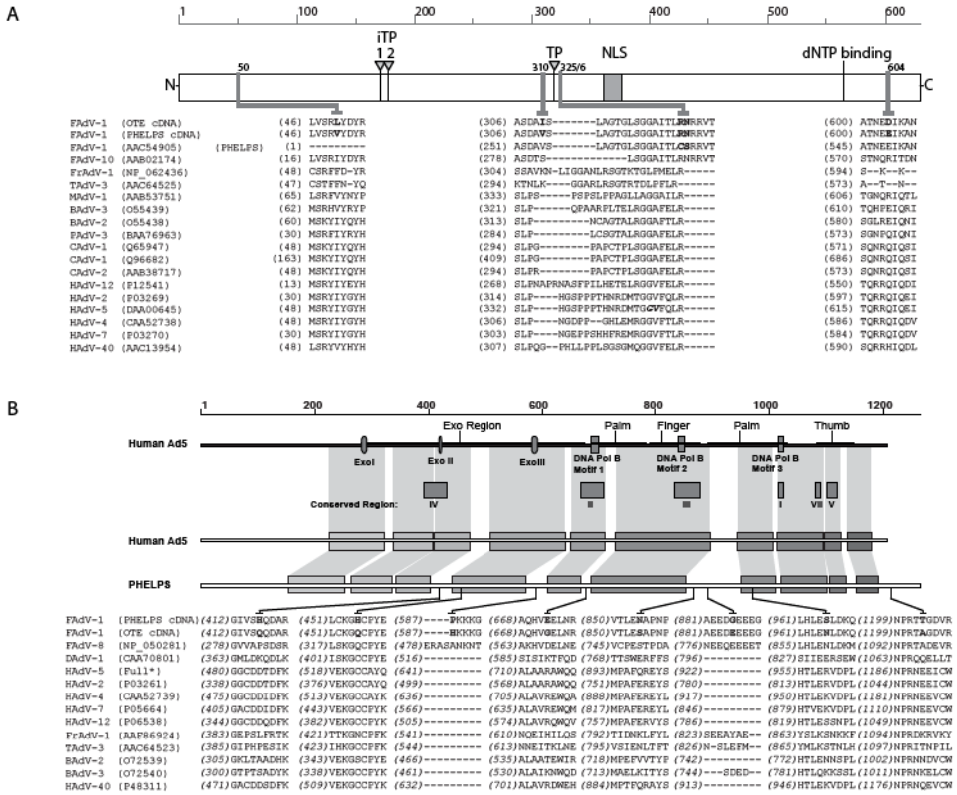

Figure 4. *Differences between PHELPS and OTE in the amino-acid sequences of pTP and Pol. Fragments encoding the pTP and Polymerase genes from PHELPS and OTE have been isolated by PCR, sequenced and compared on amino-acid levels. Amino-acid differences between PHELPS and OTE were confirmed by direct sequencing the genomic DNA. (A) Differences between PHELPS and OTE pTP sequences were aligned against publicly available Fowl (F), Frog (Fr), Turkey (T), Murine (M), Bovine (B), Porcine (P), Canine (C) and Human (H) pTP sequences. Protease-cleavage sites have been annotated by iTP1, iTP2 and TP as well as the amino acid bound to the 5' end of the viral genome ("dNTP binding"). The TP-cleavage site in the HAdV-5 sequence is indicated in bold/italic.*
*(**B**) PHELPS Polymerase was mapped against human HAdV-5 Polymerase by the macaw similarity align algorithm (pair wise cut-off: 33, score matrix: Blosum62). Significant similar blocks are equally shaded. Conserved domains in adenovirus Polymerase have been identified for HAdV-5 to identify similar functions in PHELPS Polymerase. Differences in OTE are aligned against know adenovirus Polymerase sequences. (*) The HAdV-5 Pol sequence is based on gi:118844 with the amino acids derived from exon 1 as described [31,32].*

replicon vectors [37] that contain a CMV-*GFP* expression cassette flanked by the 802 bp of the left terminus of the genome and the last 1154 bp of the right end of the genome of OTE. The mini-replicon is flanked by *Bam*HI restriction sites to release it from the backbone, leaving only a single additional nucleotide 3' of the normal genome end of the template strand. Three versions of the mini-replicons were generated: the miniOTE-C replicon contains the normal OTE termini, reading 5'-CATCATC, the miniOTE-G replicon harbours OTE ITR sequences, with the PHELPS sequence 5'-GATGATG at positions 1-7 and miniPHELPS-G replicon that contains the wtPHELPS ITRs. To study whether OTE could facilitate replication of the mini-replicons, LMH cells were infected with OTE. Six hours post-infection, the cells were washed and *Bam*HI-digested linear miniOTE-C and miniOTE-G replicons were introduced into the cells. Southern

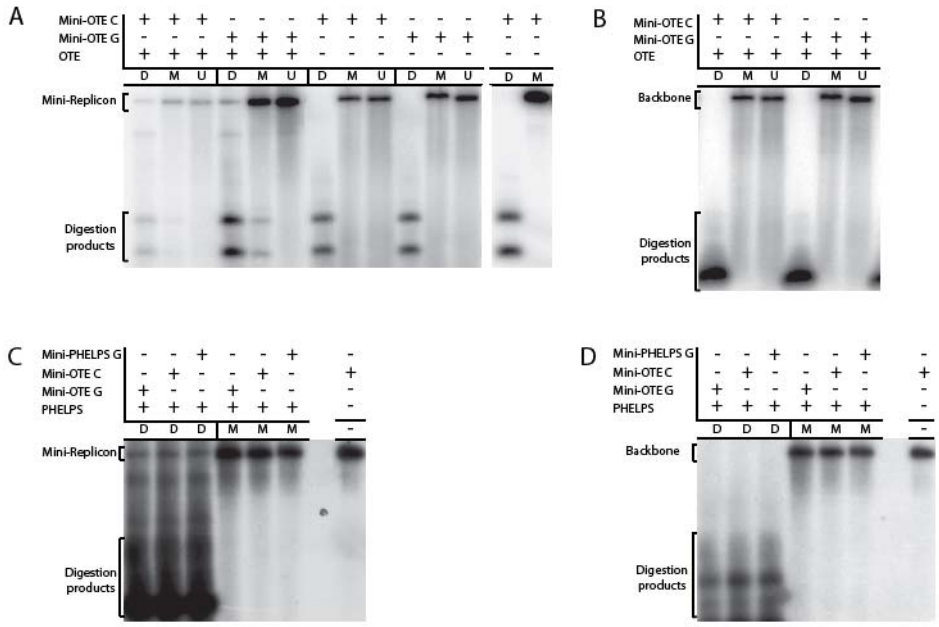

Figure 5. *Replication of mini-replicons by OTE and PHELPS. LMH cells were infected with OTE and six hours post infection, washed, and transfected with miniOTE-C or miniOTE-G plasmids digested with BamHI. The HIRT DNA-isolation method was used to extract low molecular weight DNA from the LMH cells 54-hours post infection. The isolated DNA was digested with DpnI (D, digests input DNA), MboI (M, digests replicated DNA) and analysed by Southern analysis (U=untreated). As a control, LMH cells were transfected with BamHI digested miniOTE-C or miniOTE-G without co-infection. (**A**) The mini-replicons were detected with a radiolabelled probe for the GFP gene. In the last two lanes, miniOTE-C plasmid digested with BamHI was loaded to indicate the size of the mini-replicon fragment. (**B**) To confirm full digestion by DpnI, a probe for the Amp gene was used to detect the miniOTE backbone. (**C**) Replication of mini-replicons with OTE ITR's with terminal C and G's (miniOTE-C and miniOTE-G), and miniPHELPS-G replicons based on PHELPS ITRs in cells infected with PHELPS. Minireplicons were detected with a radiolabelled probe for the GFP gene. The last lane contains the BamHI-digested miniOTE-C plasmid. (**D**) To confirm a full digestion by DpnI, a probe for the Amp gene was used to detect the mini-replicon plasmid backbones. In the last lane BamHI-digested miniOTE-C plasmid was loaded.*

analyses were performed on *Mbo*I-, *Dpn*I- or undigested low-molecular weight DNA extracted from LMH cells 54 hours post infection, using a GFP-specific probe to detect mini-replicon DNA (Figure 5). OTE is able to replicate both miniOTE-C- and miniOTE-G replicon constructs with equivalent efficiency as indicated by the *Dpn*I-resistant band (Figure 5A). Quantification of mini-replicon signals showed that 26% and 18% of the total undigested material was *de novo* replicated for miniOTE-G and miniOTE-C respectively. No hybridizing fragment is visible when a probe is used that detects the Amp gene that resides in the vector fragment (Fig. 5B). This clearly demonstrates that the restriction-endonuclease *Dpn*I digested the unreplicated DNA to completion. In addition, it shows that the generation of the unmethylated, *Dpn*I-resistant fragments is dependent on the presence of the FAdV-1 ITR sequences. Furthermore, the expected restriction fragments could be detected in the *Mbo*I-digested lanes (Fig. 5A), further indicating digestion of unmethylated DNA. The replication of mini-replicons was strictly dependent on the presence of OTE, since a *Dpn*I-resistant band or *Mbo*I-degradation products could not be detected in DNA isolated from non-infected cells. Similar results were obtained when PHELPS-infected LMH cells were cotransfected with miniOTE-C and miniOTE-G (Fig. 5C). To exclude that differences in mobilization are

caused by the sequence differences in the ITR sequence between PHELPS and OTE, a *Bam*HI-digested miniPHELPS-G plasmid, was transfected into PHELPS infected cell. The replication efficiencies of miniOTE-C, miniOTE-G and miniPHELPS-G were similar. When the blot was hybridized with an Amp probe to detect the backbone fragments, only degradation fragments were detected in the *Dpn*I-digested lanes, confirming the complete digested of input DNA (Fig. 5D). These data demonstrate that both OTE and PHELPS can drive replication of mini-replicons harbouring PHELPS 5'-GATGATG containing ITRs, as well as mini-replicons with the OTE 5'-CATCATC ITRs. This, together with the small differences in the sequences of the *pTP* and *Pol* genes, shows that the FAdV-1 viruses have relaxed their template specificity in the initiation of DNA replication.

Discussion

The terminal sequences of the PHELPS and OTE strains of the FAdV-1 were re-evaluated. We confirmed the striking sequence divergence between PHELPS and OTE at positions 1, 4 and 7 by sequencing of PCR amplified Ad DNA. Primer-extension assays, lacking either dGTP's or dCTP's in the elongation mixture, confirmed the presence of G or C residues at position 7 in virus DNA isolated from OTE and PHELPS infected cells. No heterogeneity was detected, excluding frequent reversion or indicating the stability of the ITR sequence in both isolates. Sequence analyses demonstrated that the *pTP* and *Pol* genes from PHELPS and OTE are strongly conserved. Sequence comparison did not reveal differences that would explain the distinct template usage. This led us to the hypothesis that the FAdV-1 isolates do not exhibit distinct template specificity, but rather the FAdV-1 has relaxed template specificity.

In Ads two mechanisms contribute to the stability of the DNA sequence at the origin of replication. Correct template replication depends on error-free replication. The proof-reading activity of the Ad Pol ensures faithful replication of the Ad genomes. However, due to the presence of pTP at the 5'end of the synthesized strand, proof reading fails in the first 8 to 12 bp of the Ad ITR [38]. To correct for deletions or mismatches Ad utilizes a jumping back mechanism. Small deletions of 1 or 2 nucleotides can be corrected in this way without compromising replication, as was shown by King and Van der Vliet [24]. However, mutations of nt 6 (A to C) or nt 3 (A to C) in the template strand resulted in a clear reduction of elongation efficiency in in-vitro assays. In contrast, mutation of nt 1 (G to A) did not affect replication efficiency. This suggests that the jumping-back mechanism can correct small deletions but can prevent mismatch incorporation at least at position 3 and 6. The relevance of the jumping-back mechanism in preventing elongation of mismatched DNA *in vivo* remains to be determined. Nevertheless, all known Ads contain a C residue at the 5'end of their ITR's, with the FAdV-1 isolates PHELPS and KUR as the sole exceptions. This suggests another mechanism to prevent mutation of the nucleotides at 1, 4 and 7. *In-vitro* replication-initiation experiments demonstrate that template DNA where nucleotide 4 is changed (C to A) did result in pTP-C formation (possibly initiated on nt. 7) but failed to generate pTP-T complexes [24]. Furthermore no elongation occurred on this template. Therefore, HAdV-5 seems to have two independent mechanisms to ensure ITR integrity. On the one hand, the template-dependent replication and jumping-back mechanism ensures generation of *bona fide* top-strands. In addition, the preference for binding dCMP of the pTP/Pol complex during replication initiation contributes to preventing mutations in the origin of replication.

This observation raised the question how the different ITRs in the otherwise closely

related strains PHELPS and OTE could have occurred. Obviously, the pTP-Pol complex derived from PHELPS must be able to bind dGMP as a substrate during initiation where this complex in OTE uses dCMP and conforms to the conventional Ads initiation sites. To test if this could explain the template specificity, we sequenced the *pTP* (both OTE and PHELPS) and *Pol* (OTE) genes, determined the splice sites for OTE pTP and Pol and compared both *OTE* and *PHELPS* sequences with the published PHELPS sequence. Like in HAdV-5, both pTP and Pol mRNAs are spliced and share the splice-donor site. The OTE pTP splice-acceptor site is located upstream of the pTP ORF as annotated for PHELPS, extending the pTP sequence with 55 amino acids. These additional amino acids have been shown to be essential for the biological activity in HAdV-5 pTP. Since the additional amino acids share a conserved motif [A]-[RHD]-[L]-[T]-[GN]-[Q] with other pTP's, the amino-terminal extended part is most likely important for the biological activity of OTE and PHELPS pTP. The splice acceptor site in OTE Pol is located 159 bp upstream of the annotated Pol ORF in PHELPS. The ATG translation-initiation codon in the upstream exon cannot be used for the translation of the Pol message, as it is employs another reading frame. Therefore, other than in HAdV-5 Pol, translation must initiate at the next start codon, located 208 bp downstream of the splice-acceptor site, resulting in an additional 134 aa compared to the Pol ORF annotated for PHELPS. The additional sequences do not code for domains conserved with other Ad Pol proteins. The splice sites for pTP and Pol as well as the alternative start codon of Pol confirm the predictions that Davison and colleagues made for PHELPS [39].

Sequence comparison of our PHELPS pTP gene with the published PHELPS sequence revealed 2 amino-acid differences. These amino acids are identical in OTE pTP. Comparison of PHELPS and OTE pTP revealed only two amino-acids changes. PHELPS Val^{50} and Asp^{604} were changed in OTE to Leu and Glu, respectively. These amino-acid differences have similar chemical properties or are located in the precursor part of the protein and therefore, most likely, do not cause substrate specificity. The striking similarities of the OTE and PHELPS pTP and Pol genes, suggest a relaxed sequence specificity rather than a distinct specificity of the pTP/Pol complex at replication initiation.

To test this hypothesis, we constructed the mini replicons miniOTE-C containing wtOTE ITRs and miniOTE-G where nt 1, 4 and 7 in the top strand of the ITR have been replaced with G's. Replication of linearized mini-replicon constructs was tested in a replication assay, that detects the absence of *dam* methylation in *de novo* replicated DNA. The results confirmed the ability of OTE and PHELPS to replicate ITRs starting with cytidine as well as guanidine residues with equal efficiencies. This replication was independent of the minor differences between PHELPS and OTE ITR sequences and specific for the mini-replicon constructs since a control plasmid lacking pTP/Pol binding domains, did not replicate.

The relaxed template specificity of the pTP/Pol complex is a pre-requisite for alterations in the FAdV-1 ITR to occur, but can only partially explain the altered ITR in PHELPS. Without the relaxed template specificity of the pTP/Pol complex, changes in the origin of replication are not possible. Therefore, the relaxation of the substrate specificity in the FAdV-1 replication machinery should have preceded the generation of the ITR sequences present in PHELPS and KUR.

The relaxed specificity of the FAdV-1 may be exploited for the generation of mobilisation-resistant adenoviral vectors for gene therapy. Vectors based on human Ads, in which the C residues in positions 1, 4 and 7 of the top strand are replaced with G residues would be resistant to mobilization by wild-type Ads [40].

Indeed, transfection of HAdV-5 vectors that harbour the sequence 5'-GATGATG at their genome ends did not result in the formation of plaques as these genomes are unable to replicate in helper cells. In contrast, wild type-ITR containing controls readily formed plaques and induced CPE (Rademaker, Van den Wollenberg, Hoeben, unpublished data), underlining the feasibility of this approach.

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CHAPTER 4

Adenoviruses with Mutant Inverted Terminal Repeats Replicate on Helper Cells Synthesizing a Hybrid FAdV-1 PHELPS / HAdV-5 Terminal Protein Precursor

Adenoviruses with mutant inverted terminal repeats replicate on helper cells synthesizing a hybrid FAdV-1 PHELPS / HAdV-5 terminal protein precursor

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Adenoviridae **(Ad) encode a precursor of the terminal protein (pTP) and a polymerase which are required for replication. We have previously shown that co-infection with replication-deficient Ad vector and wild-type Ad leads to mobilization of the vector. Here we describe studies that aim at blocking the replication of Ads by modifying replication-initiation sites in the inverted terminal repeats. Altering the terminal sequences of human Ad type 5 (HAdV-5) vectors from 5'-CATCATCAAT to 5'-GATGATGTAT, the terminal sequence found in the Fowl Adenovirus-1 (FAdV-1) isolate PHELPS, yielded mobilization-resistant Ad (mrAd) genomes. As expected, transfection of mrAd DNA into helper cells did not lead to lytic infection. For production of mrAds, we constructed a hybrid HAdV-5/FAdV-1 pTP gene (H2pTP) by replacing the region encompassing the nucleotide-binding serine residue of HAdV-5 pTP with the homologous region of FAdV-1. The rationale for constructing the hybrid pTP is exploiting the relaxed template specificity of FAdV-1 PHELPS, while retaining the interaction domains of HAdV-5 pTP with polymerase and host-cell factors. Transfection of mrAdCMV*GFP* DNA into 911 helper cells stably expressing the hybrid pTP yielded progeny viruses. In contrast, mrAdCMV*GFP* did not spread in the parental 911 cells. In conclusion, we constructed Ad vectors that can not be mobilized by wild-type Ads.**

The *Mastadenovirus* genus is the largest of the four genera in the family of *Adenoviridae* and contains all human adenoviruses (HAdVs). Adenoviruses (Ads) have three proteins which are involved in DNA replication: the precursor of the terminal protein (pTP), the polymerase (Pol), and the single-stranded DNA-binding protein (DBP) [1]. Of these, pTP and Pol are involved in replication initiation [2,3]. Each Ad genome has two identical origins of replication located in the inverted terminal repeats (ITRs) [4,5]. These ITRs can be functionally divided in an essential region containing the terminal direct repeat region, a pTP/Pol binding domain [6,7], and an auxiliary region that enhances replication [8-10]. The guanidine residues at position 1, 4 and 7 (G_1, G_4 and G_7) in the HAdV-5 direct repeat region (3'-GTAGTAGTTA) act as potential replication-initiation sites [11]. During replication initiation, the pTP/Pol complex interacts with the Ad genome end so that pTP can act as a primer for DNA synthesis [12-14]. Replication initiation by pTP/Pol is enhanced by the interaction with cellular transcription factors NFI and Oct-1, interacting with Pol and pTP respectively [15,16 17 18].

During replication initiation, Pol uses G_4 as its main initiation site and binds a cytidine (dCMP) residue to Serine[580] in pTP [19]. When DNA synthesis proceeds, a tri-nucleotide complex (pTP-CAT) is generated. Subsequently, this pTP-CAT complex jumps back 3 nucleotides so the CAT residues pair

with the terminal 3'-GTA residues [11]. After jumping back, pTP and Pol dissociate and elongation can proceed [20]. DNA elongation in vitro can proceed to some extent in the absence of DBP, however for efficient replication DBP is required [15,21]. Among other functions, the DBP protein acts as helicase, unwinding the double-stranded Ad genome upstream of Pol [22,23].

Whereas the genome organization and GC content are rather variable within the different Ad genera, the repeat region and pTP/Pol-binding sites in the ITR are strongly conserved. The repeat sequences in the ITRs can differ in length from 2 bp to 4 bp. In almost all Ads, the 5' termini of the genomes start with cytidine residues. In vitro studies with HAdV-5 pTP showed that pTP preferentially binds dCMP [11]. The preference of pTP for this nucleotide explains the common occurrence of cytidine (C) residues in the direct repeat region of the ITRs. Only two exceptions to the presence of the conserved C residues are known. In FAdV-1, two isolates, PHELPS (gi:9628835, gi:33694876) and KUR (gi:209935) have been described to start their genome with guanidine residues at positions 1, 4 and 7 of the top strand. However, this feature is not conserved in the FAdV-1 since the highly homologous isolate OTE (gi:210032,gi:210033) was shown to start with cytidine residues [24,25]. Comparison between PHELPS and OTE pTP did not reveal obvious differences in pTP or Pol that could explain the sequence divergence at the origin of replication (Rademaker et al., 2006). In the same study, we demonstrated that OTE minireplicons containing 'C' ITRs as well as mutants in which the cytidine residues at position 1, 4 and 7 of the 5' strand were replaced by "PHELPS" guanidine residues could be replicated upon infection with FAdV-1 OTE or FAdV-1 PHELPS.

Here, we describe the generation of HAdV-5-derived vectors, which we called mobilization-resistant Ads (mrAds) in which we mutated the terminal sequences from 5'CATCATCAAT to 5'-GATGATGTAT. We demonstrate that these mutations completely block the replication of these vector genomes in the presence of HAdV-5 pTP. However, a pTP hybrid (H2pTP) carrying the serine-binding region from the FAdV-1 PHELPS allowed replication the mrAd vector genome, as judged by the formation of plaques on 911-H2pTP helper cells. These observations provide a basis for the application of adenovirus vectors with mutated origins of replications that are resistant against mobilization by wild-type Ads.

Material and Methods

Plasmids and viruses constructs. For the generation of pmrAd-GFP, variants with altered left and right ITR have been generated with mutation PCR. The plasmid pShuttle [26] was used as template in a PCR using the primers CLITR-s and CLITR-as for the synthesis of the mutated left ITR (5-GATGATGT) and CRITR-s and CRITR-as for the mutated right ITR (5-GATGATGT). The alterations have been underlined. The left ITR was digested with XmnI and KpnI and cloned in the 6.3 kb KpnI/AvrII pShuttle fragment. The right ITR was digested with AvrII and BamHI and cloned into the 6.3 kb BamHI/XmnI pShuttle fragment. The ITRs of pmrShuttle have been sequenced to confirm the presence of the mutations at nucleotide 1, 4, 7 and 8, and to exclude the presence of other mutations in the PCR fragments. The 1.5 kb eGFP-expression cassette was isolated from pAdTrack by digesting with HpaI

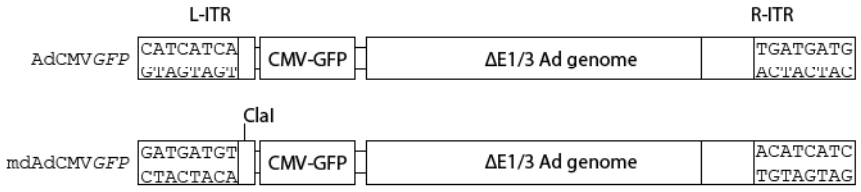

Figure 1. *Schematic representation of the AdCMVGFP and mrAdCMVGFP genomes. Differences between both genomes located at nucleotides 1, 4, 7 and 8 of both ITRs and the additional ClaI site just outside the left ITR of mrAdCMVGFP, are indicated.*

and XhoI, and the fragment was cloned into the 6.6 kb BglII/XhoI digested prdShuttle and pShuttle plasmids, resulting in prdShuttle-GFP and pShuttle-GFP respectively.

For the generation of recombinant Ad vectors, the pmrShuttle-GFP and pShuttle-GFP were digested with PmeI and introduced into E.coli BJ5183 containing the pAdEasyI plasmid, and homologous recombinants were isolated as described [26,27]. The resulting rAd plasmids were digested with PacI and introduced into 911 or 911-H2pTP cells and viruses isolated and expanded as described previously [28].

The plasmid containing the HAdV-5 pTP cDNA (pVACpTP) [29] has been kindly provided by Dr. P.C van der Vliet. The 2 kb pTP cDNA was isolated by digestion with KpnI, treated with T4 DNA polymerase and EcoRI, and cloned into the XbaI (T4 DNA polymerase treated)/ EcoRI pCDNA3.1+ fragment, resulting in plasmid pC5pTP. The PHELPS pTP fragment, cloned into the pC-H2pTP vector, has been generated by PCR with primers CPpTPh-S and CPpTPh-AS, resulting in 226 bp of the PHELPS pTP molecule replacing 217 bp of HAdV-5 pTP. The plasmid pC-H2pTP was used to make a H2pTP expressing 911 cell line (911-H2pTP).

The pCamShuttle vector was created by ligation of a 1.1 kb T4 DNA polymerase-treated PstI/Csp45I fragment derived from pGP618 (encoding the chloramphenicol acetyl transferase gene) into a 5.3 kb XmnI/BclII (T4 DNA polymerase treated) fragment of pShuttle.

Cell culture and virus propagation. The helper cell line 911 and the 911 cell line stably expressing H2pTP (911-H2pTP) were cultured in DMEM supplemented with 8% FCS, and 3 g/l glucose in a 5% CO_2 atmosphere at 37°C.

Recombinant Ads were generated by transfection of 5 µg PacI digested pAd-GFP or pmrAd-GFP plasmid into helper cells (911 or 911-H2pTP) using JetPei (Polyplus Transfection, Illkirch, France). Approximately 14-days post transfection, cells were isolated in PBS+2% HS (horse serum) and freeze/thawed to release virus particles from the cells. The freeze/thaw lysate was stored at -80 ºC and used for subsequent infection.

For Ad infection, near-confluent cultures were infected with AdCMVGFP or mrAdCMVGFP, in DMEM supplemented with 2% Horse Serum (HS), and 3 g/l glucose in a 5% CO_2 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% CO_2 atmosphere at 37°C. When almost all cells showed signs of the cytopathogenic effect (CPE), cells were harvested in phosphate-buffered saline (PBS) supplemented with 2% HS, and the cells were lyzed by three cycles of freeze/ thawing to release virus particles.

Determination of Ad terminal sequences. To determine the terminal sequences of mrAdCMVGFP vectors propagated on 911 cells, viral particles were isolated from three 10-cm dishes of 911 cells infected with mrAdCMVGFP at 48 hours post infection 28. Isolated particles were incubated with Proteinase K in 0.2% SDS, 8 mM EDTA, and viral DNA was isolated by phenol/chloroform extraction, and ethanol precipitation. The viral DNA was incubated with terminal transferase (Promega, Leiden, The Netherlands) in the presence of dATP and used in a PCR using the primers oligo-dT-20 and LITR-AS-nested resulting in a 468 bp fragment. The PCR fragment was cloned in a pCR-Topo vector and used for sequence analysis.

Results

The HAdV-5 replication machinery cannot initiate replication in vitro on Ad ITR fragments or oligonucleotides containing cytidine residues at position 1, 4 and 7 in the 3'-GTAGTAGTTA-5'terminal repeat sequence. To test whether recombinant

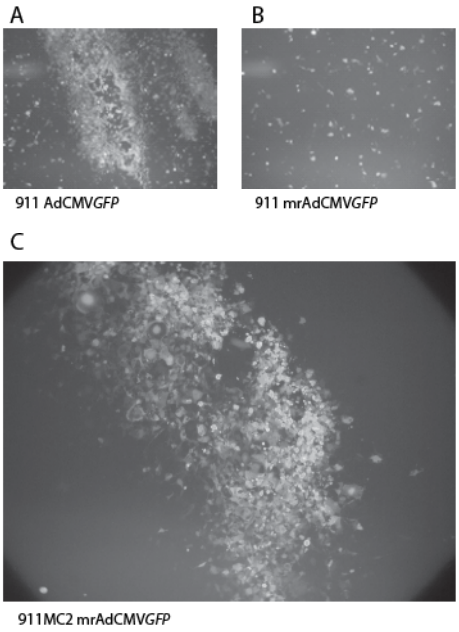

Figure 2. *GFP expression and comet formation after transfection of 911 and 911-H2pTP cells with AdCMVGFP or mrAdCMVGFP DNA. A 10-cm dish containing 911 or 911-H2pTP cells was transfected with 5µg PacI-digested pAdCMVGFP or pmrAdCMVGFP using JetPei. Viral spread was determined by the detection of GFP-expressing cells, 14-days post transfection. Comet shaped groups of positive cells are indicative for adenoviral spread in cells cultured in medium. A) 911 cells transfected with 5 µg PacI-digested pAdCMVGFP. B) 911 cells transfected with 5 µg PacI digested pmrAdCMVGFP. C) 911-H2pTP cells transfected with 5 µg PacI digested pmrAdCMVGFP.*

HAdV-5 vectors containing C residues rather than the wild-type G residues at positions 1, 4 and 7 of the template strand are able to replicate in helper cell lines, we constructed a pShuttle-based plasmid containing such mutations in both ITR's (pmrShuttle). The presence of these mutations in pmrShuttle as well as the additional *Cla*I site introduced in the left Ad terminus were confirmed by direct sequencing. To determine the transfection and infection efficiencies of this construct, a GFP gene driven by the CMV promoter was introduced (pmrShuttleCMV*GFP*). Recombinant adenoviral genomes were generated by homologous recombination with pAdEasyI and digestion with *Pac*I. Viral vectors generated with this plasmid were called mobilization-resistant Ads (mrAds). To test whether mrAdCMV*GFP* genomes are able to replicate, the *E1*-complementing cell line 911 was transfected with 5 µg of *Pac*I-digested pmrAdCMV*GFP*. As a control, we transfected plasmid DNA containing *wt*ITRs (pAdCMV*GFP*). Transfection efficiencies were similar as determined by the amount of GFP-positive cells 2-days post transfection (data not shown). Ten-days post transfection, comet-shaped plaques of GFP-positive cells were visible in 911 monolayers transfected with pAdCMV*GFP* (see figure 2A), indicative of replication and viral spread. In contrast, neither at day 10 post-transfection, nor at later time points, mrAdCMV*GFP* vector spread was detectable.

To check the integrity of the plasmid, pmrAdCMV*GFP* plasmid was recombined with p*Cam*ShuttleCMV*GFP* (see figure 3) resulting in the reintroduction of *wt*ITRs while maintaining the backbone of pmrAdCMV*GFP*. Both ITRs have been sequenced and confirmed the presence of *wt*AdV-5 ITRs. Transfection of this *Pac*I-digested plasmid into 911 cells resulted in comet-shaped GFP-positive plaques, indicative for virus spread. Growth characteristics of the recombinant virus were similar to AdCMV*GFP* in transfected 911 cells, confirming that the lack of mrAdCMV*GFP* replication was not due to inadvertent mutations in the viral backbone. Taken together, these data confirm that mutation of nt 1, 4 and 7 from C to G completely abolishes the vector's capacity to replicate.

To restore the replication capacity of the mrAd vectors, alterations in pTP are required

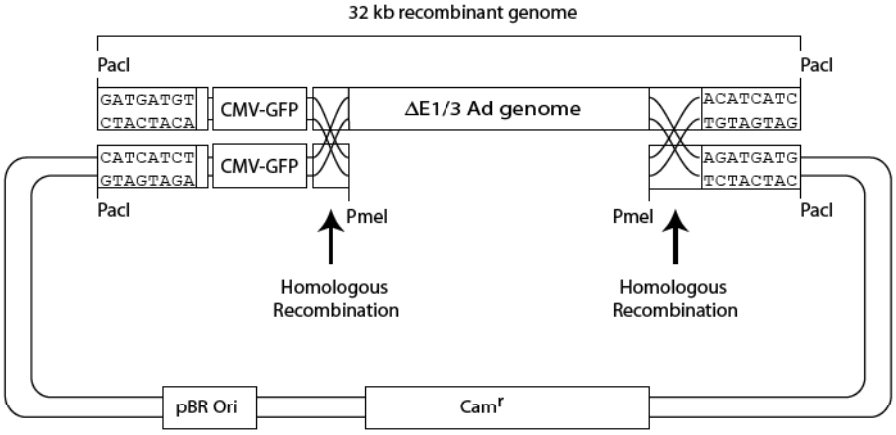

Figure 3. *Schematic representation of the re-introduction of wtITRs by homologous recombination. The plasmid pmrAdCMVGFP was digested with PacI. The 40-kb band was isolated and with PmeI-digested pCamShuttleCMVGFP co-electroporated into E.coli BJ5183 bacteria. Bacteria were cultured on Chloramphenicol-containing agar plates, selecting for those bacteria that harbored recombined plasmids containing the wtITRs while retaining the mrAdCMVGFP viral backbone.*

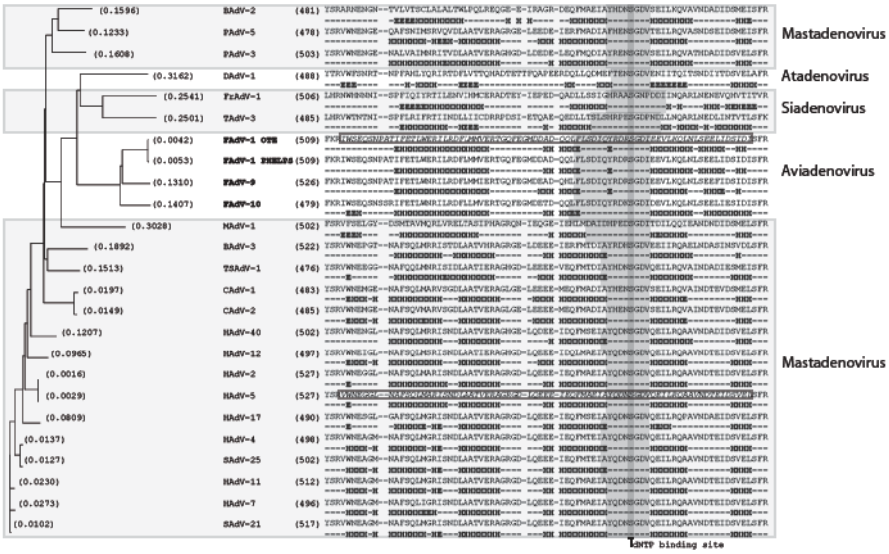

Figure 4. *Structure prediction on the region surrounding the nucleotide-binding Serine of pTPs. A structure prediction of the available pTP aa sequences was performed using the NNpredict algorithm (http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html). The predicted secondary structure was projected on the aligned pTP sequences. Both the aa sequence in HAdV-5 pTP that has been replaced and the corresponding FAdV-1 Phelps pTP sequence is indicated in italic script and has been boxed. The nucleotide binding serine has been shaded dark gray and the serine containing region flanked by the two helixes is shaded light gray. (H = helix element, E = β-strand element and - = turn element).*

to accept dGTP as substrate. The only known Ads able to initiate replication with dGMP are the Fowl Adenovirus 1 (FAdV-1) strains PHELPS and KUR. We have previously shown that the FAdV-1 PHELPS and OTE have relaxed their template specificity, allowing replication initiation with either dGMP or dCMP as first nucleotide [25]. Because the crystal structure of pTP is unknown, we performed a structure prediction on a region surrounding the dNTP binding serine for all currently known pTPs (Figure 4), using the NNpredict algorithm [30]. From the predicted structure it appears that the nucleotide binding serine is flanked by two helixes. The region between the helixes is 8 to 9 amino acids in most members of the genus *Mastadenovirus*. In the fowl adenovirus-1 (FAdV-1), a member of the *Aviadenovirus* genus, this region spans approximately 14 aa. To test whether this larger region affects the specificity, we replaced a 76 aa fragment containing the nucleotide-binding serine flanked by both helices (see figure 4, italic font) in HAdV-5 pTP by the homologous region from FAdV-1. By maintaining the majority of pTP from HAdV-5 we reduced the risk of disrupting domains of pTP that interact with Pol and Oct-1. The gene encoding this hybrid pTP called *H2pTP* was cloned in the pCDNA3.1+ expression vector.

To test whether H2pTP could facilitate replication of mrAdCMV*GFP* vectors, we generated 911 cells stably expressing the H2pTP gene (911-*H2pTP*). The pTP protein was present in all cells and localized both in the nucleus and the cytoplasm (Figure 5). These cells were transfected with 5 µg *Pac*I-digested pAdCMV*GFP* or pmrAdCMV*GFP*. Transfection efficiencies were equal, as determined by the amount of GFP-positive cells, assessed two-days post transfection (data not shown). Ten-days post transfection, viral spread in 911-*H2pTP* monolayers transfected with pAdCMV*GFP* was noted (data not shown) as well as in wells exposed to pmrAdCMV*GFP* DNA (figure 2B). To exclude virus contamination,

Figure 5. *Detection of 911-H2pTP by immune fluorescence microscopy. The (A) 911-H2pTP and (B) 911 control cells were cultured on glass plates, fixed with methanol/acetic acid and stained by rapTP/Pol [33] and FITC-labeled gar-Fab.*

both AdCMV*GFP* and mrAdCMV*GFP* viruses were propagated on 911-*H2pTP* cells and viral DNA was isolated. A PCR using the primers CLITR-S and LITR-AS-nested was performed to amplify the L-ITR. The PCR product was digested with *Cla*I and size fractionated on an agarose gel. For AdCMV*GFP*, only one fragment was visible while *Cla*I -digestion of mrAdCMV*GFP* revealed two fragments (data not shown and figure 6). Therefore we conclude that the 911-*H2pTP* cells support replication of mrAdCMV*GFP* while the parental 911 cells do not.

The mrAdCMV*GFP* viruses propagated on the 911-*H2pTP* cell line should not be able to replicate on cells in the absence of H2pTP. Therefore, we infected 911 cells with mrAdCMV*GFP* that was propagated on 911-*H2pTP* cells. Two-days post infection, the 911 monolayer started to show Cyto Pathogenic Effect (CPE), indicative of virus replication. Further examination proved that all cells were expressing GFP (data not shown). This excludes contamination with *wt*Ads. Since both H2pTP in the helper cell line and the pTP gene in the viral backbone are homologous around the Ser^{580} region, we must exclude that the vector acquired the H2pTP gene from the cell lines by homologous recombination. Therefore, a 396-bp region in the pTP gene was amplified using PCR with the primers H2pTPseq-S and H2pTPseq-AS and cloned in a pCR2.1-Topo vector. Sequence analysis of the PCR fragment revealed the presence of only *wt*AdV-5 pTP sequences. This excludes the possibility that the mrAdCMV*GFP* vector acquired the H2pTP gene by homologous recombination.

As an alternative explanation, we determined whether the mrAdCMV*GFP* ITR sequences have reverted to the *wt*ITR sequences. To that end 3' termini of viral mrAdCMV*GFP* DNA was poly-adenylated with terminal-transferase and used in a PCR with the primers poly-dT and LITR-As-nested to PCR amplify the mrAdCMV*GFP* left ITR. The left ITR was sequenced (Figure 6) and showed the presence the Cytidine residues at position 1, 4 and 7 of the 5' terminus of the left ITR. In addition to the cytidine residue, also the thymidine residue at position 8 was reverted to an adenine residue. The *Cla*I site, which is unique in mrAdCMV*GFP*, was still present in the vector, again formally excluding contamination with AdCMV*GFP*. From these data we conclude that HAdV-5 vectors in which the direct repeat sequence 5'-CATCATCA has been changed to 5'-GATGATGT do not replicate in 911 cells. Upon expression of the hybrid PTP gene

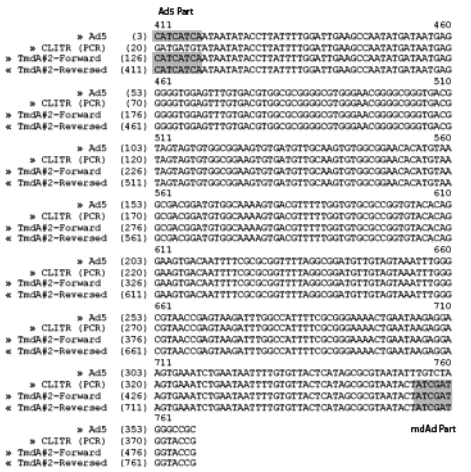

Figure 6. *Left ITR sequence of mrAdCMVGFP virus generated on 911-H2pTP cells and propagated on 911 cells. The first generation of mrAdCMVGFP virus was generated on 911-H2pTP cell line. Virus from this cell line was isolated and passed onto 911 cells for propagation. Fourth-passage virus (third passage on 911) was CsCl purified and used to isolated DNA. The 3'-termini have been extended using terminal transferase and dATPs and was used in a PCR to amplify the left ITR using the primers oligo-dT-20 and LITR-AS-nested. The PCR fragment was cloned and sequenced.*

H2pTP, these vectors do replicate, but in the presence of a *wt* pTP gene, revertants with a *wt* ITR sequence rapidly overgrow the mrAd vector population.

Discussion

Classical recombinant Ad vectors do not replicate due to the deletion of essential viral genes. Their replication deficiency is an important safety feature for clinical applications, as it ensures resticted transgene expression and limits potential toxicity. Co-infection of vector-transduced cells with *wt*Ads or replication-competent adenovirus (RCA) generated during vector production can complement the deficiency, resulting in replication and mobilization of the rAd vector [31]. This may result in aggravation of the host immune response and other unpredictable side effects.

In HAdV-5 initiation, dCMP is covalently bound to pTP, which serves as a protein primer for DNA synthesis. Modifications in the template strand of the terminal direct repeat in the ITR blocks replication initiation. We constructed a mobilization-defective HAdV-5 vector containing cytidine residues at position 1, 4 and 7 of the template strand, instead of guanidine residues. Although transfection efficiencies were similar, *Pac*I-digested pmrAdCMV*GFP* did not yield progeny virus when transfected to 911 cells. In contrast, the pAdCMV*GFP* carrying *wt*ITR sequences yielded recombinant viruses at the expected frequency. To exclude that lack of replication is caused by inadvertent alterations generated in the backbone of mrAdCMV*GFP* during production of the plasmids, the ITRs of mrAdCMV*GFP* were converted to *wt*ITR sequences by homologous recombination while maintaining the backbone sequences. Transfection of the reverted mrAdCMV*GFP* plasmids did result in the formation of progeny virus, thus demonstrating that the alteration of the ITR sequences causes a block in the formation of progeny virus.

To enable replication of these mrAdCMV*GFP* vectors, changes in pTP are required to relax the template specificity. The pTP of FAdV-1 isolated PHELPS and OTE served as a model for such pTP molecules. The FAdV-1 family is currently the only known family that contain isolates that start with guanidine residues in the 5' termini of the ITR (isolates PHELPS and KUR). Therefore, PHELPS pTP can initiate replication with dGTPs. The FAdV-1 strain OTE starts with cytidine residues in the 5' termini. Our previous study revealed that the pTP molecules of both PHELPS and OTE are capable of initiating replication with both dCTP and dGTP [25]. Since no structure information of pTP is known, we performed an NNpredict structure prediction on all known pTP molecules. For all pTPs, the nucleotide-binding Serine is flanked by a helical structure. For all HAdVs the distance between the two helixes is 8 or 9 amino acids. With the exception of the murine AdV-1, this is true for all known *Mastadenovirus* pTPs. A clear difference is seen within the *Aviadenovirus* genus. The FAdV-1 isolates PHELPS and OTE as well as the FAdV-9 and 10 serotypes contain a stretch of 14 amino acids between the helixes. It is tempting to speculate that this difference is correlated with the relaxed substrate specificity documented for the FAdV-1 isolates.

To test this hypothesis, we constructed H2pTP, a HAdV-5 based pTP containing a 76 aa fragment of OTE including both helixes surrounding the nucleotide binding serine. The majority of the pTP remained of HAdV-5 origin to minimize the risk of losing interaction domains with Oct-1 and Pol. The *H2pTP* was stably transfected into 911 cells (911-*H2pTP*). Immunofluorescence confocal microscopy showed that the protein was present in both the nucleus and the cytoplasm of all cells. Indeed, Ad vectors with 5'-GATGATGT containing ITRs, which do not replicate in 911 cells, readily formed comets on 911-*H2pTP* lines. These data suggest that H2pTP, in contrast to HAdV-5 pTP, can initiate on 3'-CTACTACA

Sequence entry	Chromosome	Start (nt)	Sequence	End (nt)
>ref|NT_008046.15|Hs8_8203	chromosome 8	19070182	.ATCATCAATAATATACC	19070166
>ref|NT_011109.15|Hs19_11266	chromosome 19	17182487	..TCATCAATAATATACC	17182502
		17183105	..TCATCAATAATATACC	17183120
		17188602	..TCATCAATAATATACC	17188617
		17230353	..TCATCAATAATATACC	17230338
		17230944	..TCATCAATAATATACC	17230929
>ref|NT_006238.10|Hs4_6395	chromosome 4	8984747	..TCATCAATAATATACC	8984732
		8985335	..TCATCAATAATATACC	8985320
		8990769	..TCATCAATAATATACC	8990754
		8991357	..TCATCAATAATATACC	8991342
		8996932	..TCATCAATAATATACC	8996917
		8997520	..TCATCAATAATATACC	8997505
		9003087	..TCATCAATAATATACC	9003072
		9003675	..TCATCAATAATATACC	9003660
		9009367	..TCATCAATAATATACC	9009352
		9009955	..TCATCAATAATATACC	9009940
		9013687	..TCATCAATAATATACC	9013702
		9014275	..TCATCAATAATATACC	9014290
		9019683	..TCATCAATAATATACC	9019698
		9020271	..TCATCAATAATATACC	9020286
		9025849	..TCATCAATAATATACC	9025864
		9026437	..TCATCAATAATATACC	9026452
		9032894	..TCATCAATAATATACC	9032909
		9033482	..TCATCAATAATATACC	9033497
>ref|NT_037645.2|Hs4_37649	chromosome 4	26599	..TCATCAATAATATACC	26614
		27187	..TCATCAATAATATACC	27202
>ref|NT_005612.14|Hs3_5769	chromosome 3	77228698	..TCATCAATAATATACC	77228683
>ref|NT_005403.15|Hs2_5560	chromosome 2	12347785	..TCATCAATAATATACC	12347800
>ref|NT_005058.14|Hs2_5215	chromosome 2	1649096	..TCATCAATAATATACC	1649081
		1654999	..TCATCAATAATATACC	1654984
>ref|NT_011786.15|HsX_11943	chromosome X	27414327	...CATCAATAATATACC	27414313
>ref|NT_011255.14|Hs19_11412	chromosome 19	5999370	...CATCAATAATATACC	5999384
>ref|NT_033899.7|Hs11_34054	chromosome 11	37198980	...CATCAATAATATACC	37198994
>ref|NT_008413.16|Hs9_8570	chromosome 9	28155979	...CATCAATAATATACC	28155993
>ref|NT_006713.14|Hs5_6870	chromosome 5	28881507	...CATCAATAATATACC	28881521
>ref|NT_022184.14|Hs2_22340	chromosome 2	66962359	...CATCAATAATATACC	66962373
>ref|NT_022135.14|Hs2_22291	chromosome 2	4424023	...CATCAATAATATACC	4424009
>ref|NT_037485.3|Hs1_37489	chromosome 1	251418	...CATCAATAATATACC	251404

***FIGURE** 7. Potential Ad replication initiation sites in the human genome based on the HAdV-5 repeat region and pTP/Pol binding site. The putative replication initiation sites based on HAdV-5 are shaded light gray while the consensus pTP/pol binding sequence is shaded dark grey. Hits are grouped by reference id and sequence position. The sequences were retrieved by blast searching for the HAdV-5 ITR in the human genome reference database (using BlastN, Expect: 10, Filter: None; http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html).*

templates. To exclude a contamination with AdCMV*GFP* virus, we passed these viruses three times on 911-*H2pTP*, isolated the virus DNA, and used this to amplify the left ITR by PCR. The presence of the *Cla*I site which was introduced in the left ITR of mrAds, was demonstrated by digestion analysis on the PCR product confirming the mrAdCMV*GFP* origin of the replicating virus.

To formally prove that H2pTP is required for the replication of mrAdCMV*GFP* vectors, 911 cells were infected with mrAdCMV*GFP*. Unexpectedly, three days post infection CPE started to be apparent, indicative for replication. Viruses isolated from these 911 cells could be expanded and propagated, demonstrating that viruses produced on 911-*H2pTP* lost their restriction to only replicate in the presence of H2pTP. This was not due to the vector acquiring the H2pTP gene by homologous recombination since sequence analysis of the pTP gene in this vector only revealed the presence of the *wt*HAdV-5 pTP gene.

To test whether the ITR sequences had reverted to wildtype, viral DNA was isolated and extended with terminal transferase. A PCR amplified fragment, which contained the entire left ITR, was cloned and sequenced. Sequence analysis revealed the presence of the *Cla*I site, like previously shown by restriction analysis, demonstrating

that the viral DNA was of mrAdCMV*GFP* origin. Strikingly, the Cs at position 1, 4 and 7 of the template strand of the ITR had all reverted to G's. Also, the A at position 8 has been reverted to T, resulting in a sequence identical to the *wt*HAdV-5 ITR. Although Pol has no proofreading activity in this region of the ITR [32], the jumping back mechanism was suggested to prevent mis-incorporation at these sites. Furthermore, the chance of 4 mismatch incorporations seems unlikely, although the error rate of HAdV-5 Pol lacking proofreading is not known. A last possibility that could explain the reversal of the ITRs is either homologous recombination of pseudo Ad ITR sequences in the human genome, or replication initiation on these genomic pseudo ITR sequences (Figure 7). It is to be expected that the reversion would be less prominent if the *wt*HAdV-5 Pol gene is deleted from the backbone of the vector gene. Nevertheless, our data describe a hybrid pTP that facilitates replication-initiation at origin sequences that can not be recognized by *wt*HAdV-5 sequences.

Acknowledgements
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CHAPTER 5

Towards integrating vectors for gene therapy: expression of functional bacteriophage MuA and MuB proteins in mammalian cells

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Towards integrating vectors for gene therapy: expression of functional bacteriophage MuA and MuB proteins in mammalian cells

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Bacteriophage Mu has one of the best studied, most efficient and largest transposition machineries of the prokaryotic world. To harness this attractive integration machinery for use in mammalian cells, we cloned the coding sequences of the phage factors MuA and MuB in a eukaryotic expression cassette and fused them to a FLAG epitope and a SV40-derived nuclear localization signal. We demonstrate that these N-terminal extensions were sufficient to target the Mu proteins to the nucleus, while their function in *Escherichia coli* was not impeded. *In vivo* transposition in mammalian cells was analysed by co-transfection of the MuA and MuB expression vectors with a donor construct, which contained a miniMu transposon carrying a Hygromycin-resistance marker (Hyg^R). In all co-transfections, a significant but moderate (up to 2.7-fold) increase in Hyg^R colonies was obtained if compared with control experiments in which the MuA vector was omitted. To study whether the increased efficiency was the result of bona fide Mu transposition, integrated vector copies were cloned from 43 monoclonal and one polyclonal cell lines. However, in none of these clones, the junction between the vector and the chromosomal DNA was localized precisely at the border of the Att sites. From our data we conclude that expression of MuA and MuB increases the integration of miniMu vectors in mammalian cells, but that this increase is not the result of bona fide Mu-induced transposition.

For gene therapy of hereditary diseases, long-term expression of the transgene is essential. Integration of the transgene into the DNA of the target cells is required for persistence of the transgene upon cell division. Recent studies on transposable elements have revealed their feasibility as tools in mammalian genetic research and gene therapy [1-4]. Although several prokaryotic systems have been applied successfully in mammalian cells [5,6], the applicability of a prokaryotic transposase was never reported.

In the prokaryotic field, bacteriophage Mu has one of the best characterized transposition machineries, which is unequalled in its efficiency [7]. In addition, the 37-kb bacteriophage genome is one of the largest transposable elements which should provide ample space for exogenous sequences. Mu transposition requires the transposase MuA, a phage-encoded 75-kDa protein which, as a monomer, binds DNA in a sequence-specific manner [8,9]. The DNA strand cleavage and joining reactions are established by a higher-order protein–DNA complex, the so-called Mu transpososome. This complex consists of a tetramer of MuA proteins, which binds to the two ends of the Mu genome, the so-called L and R attachment sites (L att and R att, respectively). An internal activation

sequence (IAS) is involved in the initial formation of this complex as well, and enhances transposition nearly 100-fold [10]. The active transpososome performs a single-stranded cleavage of the Mu DNA just outside the L and R att sites in such way that 3′-OH groups of the transposable element are exposed. These 3′-ends are subsequently joined to the 5′-ends of the target DNA, which has been cleaved by a 5-bp staggered cut. The resulting DNA structure is a common intermediate for the two transposition products: co-integration and simple insertion [11]. In each case, the staggered cut will cause a duplication of 5 bp of the target DNA enclosing the transposed element.

For efficient transposition a second Mu-encoded protein, MuB, is required. This 35-kDa protein forms a protein–protein complex with the transposase and stimulates the assembly of the transpososome [12], the cleavage of Mu DNA and the intermolecular strand transfer into the target site [13-15]. These stimulatory features cause a 100-fold increase in transposition efficiency [16]. Besides MuA, MuB and a proper DNA template, efficient Mu transposition also depends on the host-related factor Hu and on the presence of ATP and Mg^{2+} [17]. Although Hu is absent in mammalian cells, it has been suggested that the eukaryotic proteins HMG-1 and HMG-2 can replace Hu in the assembly of the Mu transposition complex [18,19]. Moreover, recent studies on *in vitro* Mu transposition showed that integration can take place in the absence of host-encoded elements and depends solely on the MuA transposase and appropriate DNA substrates [20,21]. This reduced *trans*-requirement for transposition, together with its large transposon length and its high efficiency, makes the Mu transposition machinery an appealing system to be explored for chromosomal integration of therapeutic genes after gene transfer with non-integrating adenoviral or non-viral vectors.

In this study, we describe the adaptation of the bacteriophage Mu integration machinery from its prokaryotic host to mammalian cells, in order to evaluate its feasibility as integrating entity for gene-therapy purposes

Materials and Methods

Bacterial strains and plasmids. The bacterial strains used were Stable 2 (Life Technologies, Breda, The Netherlands), INVαF' (Invitrogen, Groningen, The Netherlands), MC1061 (Stratagene Europe, Amsterdam, The Netherlands), KMBL 1164 [Δlac-proXIII, thi209, $SupE^+$](16) and KMBL 1001 [$SupE^-$], derived from W1485 [22].

Plasmid pmMu876 was derived from pGP876 [23] by removal of the genetic code for MuA and MuB by partial EcoRI digestion and re-circularization.

The construction of plasmids pSC-NLS-MuA and pSC-NLS-MuB, which contain the transposase, MuA and transposition stimulator, MuB, respectively, was performed as described below. To isolate the Mu-A coding sequence, pGP876 was digested with the restriction enzymes EcoRV and PmeI, a fragment of 2018 bp was isolated and blunted by Klenow polymerase filling-in of the sticky-ends. Subsequently, this fragment was ligated into the eukaryotic expression vector pSuperCatch-NLS [24], a derivative of pCatch-NLS, which had been digested with BamHI and filled-in with Klenow polymerase. The construct, which contained MuA under control of the cytomegalovirus promoter (pCMV), was called pSC-NLS-MuA (Fig. 1A).

The coding sequence for MuB was obtained by digestion of pGP876 with SspI and DraIII. A fragment of 1231 bp was isolated and the DraIII-originated 3′overhang was removed by exonuclease 3′→5′. This blunt-ended fragment was cloned into the BamHI-digested and Klenow polymerase-blunted pSuperCatch-NLS. The construct pSC-NLS-MuB (Fig. 1A) contained the MuB gene driven by the CMV promoter. DNA sequence analyses confirmed the integrity of the modified MuA and MuB genes in plasmids pSC-NLS-MuA and pSC-NLS-MuB.

Three donor constructs, plasmids containing a miniMu transposon, have been used in the in vivo transposition experiments (Fig. 1B). The first construct, pmMuHyg was constructed by digestion of plasmid pCep4 (Invitrogen) with NruI and partially with SalI, isolation of the 3.2-kb fragment and cloning it into SmaI-and SalI-digested pmMu876. The second donor construct, pmMuHyg-GFP, is identical to the first one, except that a GFP-marker is added outside the miniMu transposon. First, the GFP-marker was cloned into pmMu876. To that end, plasmid phGFP-S56T (Clontech Laboratories, Palo Alto, CA) was digested with MluI and BamHI, the resulting 2.3-kb fragment, which carries the complete GFP-expression cassette, was isolated and blunted with Mung Bean nuclease. This fragment was cloned into pmMu876, which had been linearized with ClaI and

blunted with Mung Bean nuclease. The Hygromycin-resistance marker (Hyg^R) was subsequently cloned into the resulting construct in the same way as described for pmMuHyg. The last donor construct, pmMuNeo was made as follows. Plasmid pmMu876 was digested with AatII and SalI and a 5927-bp fragment was isolated. In this we cloned the 3707-bp AatII-and SalI-digested fragment from the retroviral vector pBAG [25].

Immunofluorescence. The detection of the MuA and MuB proteins, both fused to a Flag epitope, was performed with a 400-fold dilution (3% BSA in PBS) of a mouse monoclonal antibody against the Flag epitope (mαFlag M2; Kodak, New Haven, CT). Fluorescein-isothiocyanate-labeled goat anti-mouse antibody (GαMFitc; Jackson Immunoresearch Laboratories, WestGrove, PA) was used as second antibody. Nuclear DNA was stained 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.

Protein analysis by western blotting. The hybrid protein products from pSC-NLS-MuA and pSC-NLS-MuB have been produced in vitro with the TNT T7 Coupled Reticulocyte Lysate System (Promega, Leiden, The Netherlands), in accordance with the manufacturer's protocol. Stably transfected 911 cells provided the hybrid proteins after in vivo expression. Cell lysate was obtained by scraping the cells in RIPA lysis buffer (25mMTris–HCl pH7.4,50mM NaCl, 0.5% Doc, 2% NP-40, 0.2% SDS). After a 10 min incubation at room temperature, the lysates were cleared by centrifugation and the protein concentration of the supernatant was measured by the Bradford protein assay.
Cell-lysate and in-vitro transcription translation samples were fractionated on a 10% SDS–PAGE gel. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) and incubated with mαFlag (Kodak) as first antibody. The second antibody was a horseradish peroxidase-labeled goat anti-mouse antibody (Brunschwig Chemie, Amsterdam, The Netherlands). The resulting protein–antibody complexes were visualized by enhanced chemiluminescence.

Complementation of MuA am and MuB am phages. Cultures of the Escherichia coli strains KMBL 1001 [Sup E^-] transformed with pSC-NLS-MuA, KMBL 1001 [Sup E^-]transformed with pSC-NLS-MuB and KMBL 1164 [Sup E^+]were diluted to an $OD_{\lambda 650}$=0.1 (10^8 cells/ml). At this concentration, 1 ml culture samples were infected by wild-type bacteriophage Mu, MuA am or MuB am at multiplicities of infection (m.o.i.) of 2–3. After phage addition, the samples were incubated at 37°C for 20 min, without perturbation. Then, the samples were diluted 1000-fold (5 µl:5 ml) and grown at 37°C. After 1 h incubation, 50 µl chloroform was added, the samples were centrifuged for 10 min at 800 g and the phage-containing supernatant was collected.
The phage titre of the supernatant was determined on

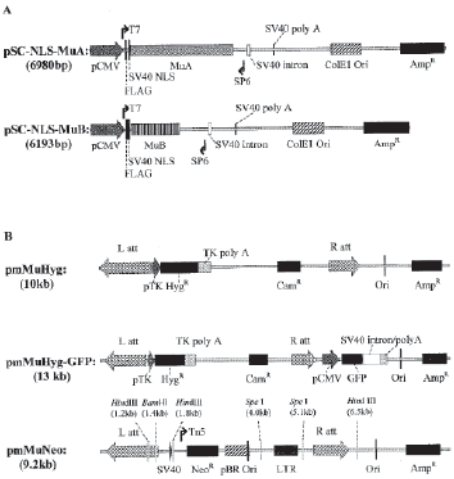

Figure 1. *Constructs used in the analysis of Mu transposition in mammalian cells.* **(A)** *Plasmid pSC-NLS-MuA contains the eukaryotic expression cassette encoding the Mu transposase, MuA, fused to a Flag epitope and SV40 NLS. The T7 RNA-polymerase transcription initiation site enables prokaryotic expression of this fusion protein. Plasmid pSC-NLS-MuB contains the eukaryotic expression cassette encoding the transposition stimulator, MuB, similar to the MuA construct.* **(B)** *The donor plasmids are characterized by the presence of a miniMu transposon, delimited by the L and R attachment domains (L att and R att). These att domains are depicted as arrows pointing to the location of the MuA-induced nick sites. The L att domain also includes the LAS region. The miniMu transposon of pmMuHyg contains a Hyg^R marker driven by a TK promoter. Donor construct pmMuHyg-GFP contains the previously mentioned miniMu transposon with Hyg^R marker and a CMV-driven GFP marker, which is located just outside this transposon. The miniMu transposon from pmMuNeo contains a Neo^R marker driven by the SV40 promoter for eukaryotic expression and by the Tn5 promoter for prokaryotic expression. This element also includes a pBR322-derived origin of replication (pBR Ori). pCMV, immediate early promoter from cytomegalovirus; pSV40, promoter from Simian Virus 40; PolyA, polyadenylation site; ColE1, origin of replication; NLS, nuclear-localization signal; Amp^R, Ampicilin-resistance marker; T7 and Sp6, RNA-polymerase transcription-initiation sites; pTK, thymidine-kinase promoter; Hyg^R, Hygromycin-resistance marker; Cam^R, Chloramphenicol-resistance marker; GFP, green fluorescence protein; LTR, long terminal repeat; Tn5, Tn5 Neo promoter. Unless indicated otherwise, the plasmids were used in supercoiled form.*

strains KMBL 1164 and KMBL 1001. The supernatant was diluted 10-or 1000-fold and 100 µl of these dilutions were added to a 1-ml culture of KMBL 1164 or 1001, respectively. The mixture was incubated for 20 min at 37°C, without perturbation. Hereafter, 3 ml top agar (1:1, agar versus LB) was added and immediately transferred to LB plates. After overnight incubation

at 37°C the plaques were counted and the titre was calculated.

Cell culture, transfection and selection. The Ad5E1-transformed human embryonic retina cell line 911 [26] and the osteosarcoma cell line U2OS were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), antibiotics and 3 g/l glucose in a 5% CO_2 atmosphere at 37°C. Transfection of 911 cells was performed by the calcium-phosphate technique [27]. U2OS cells were transfected with Fugene (Boehringer Mannheim, Almere, The Netherlands) in accordance with the manufacturer's protocol. Approximately 48 h post-transfection, selection of transfected cells was started by the addition of 150 µg/ml Hygromycin (Boehringer Mannheim) or 450 µg/ml G418 (Life Technologies) to the medium. One week post-transfection, all mock-transfected cells died and Hygromycin or G418-resistant colonies appeared. Selection pressure was then reduced to 50 µg/ml Hygromycin or 200 µg/ml G418. Solitary colonies were picked, cell lines established and chromosomal DNA isolated.

Analysis of chromosomal DNA for transposition events. Chromosomal DNA was isolated from G418-resistant colonies obtained after co-transfection of 911 or U2OS cells with the donor construct pmMuNeo, pSC-NLS-MuA and with or without pSC-NLS-MuB. To check for Mu-catalyzed integrations, the DNA flanking the att-sites was analyzed by the following procedure. For the analysis of the DNA flanking the L att site, 1 µg chromosomal DNA was digested overnight with 3 U of SpeI (Fig. 1B). After heat inactivation of the restriction enzyme, the samples were diluted to 4 ng/µl and the fragments circularised. After overnight incubation, DNA was precipitated and used for electroporation of E.coli strain Stable 2. Trans-formants containing the Neomycin-resistance marker (Neo^R) were selected by growth on Kanamycin-containing LB plates at 30°C. The resulting colonies were checked for Mu-related origin by contra-selection on Ampicilin (Amp). The Amp-sensitive colonies were further analyzed by SpeI and HindIII restriction analysis, PCR based on primers surrounding the Mu-induced nick site and, finally sequence analysis.
The same analyses were performed to study the DNA flanking the R att site, but in this case BamHI was used to digest the chromosomal DNA.

RESULTS

Evaluation of the constructs pGP876, pSC-NLS-MuA and pSC-NLS-MuB

The construct pGP876 provides all the vital elements for Mu transposition, since it contains the MuA and MuB genes and a miniMu transposon, consisting of a Chloramphenicol-resistance marker (Cam^R) delimited by the L and R attachment sites. The functionality of these elements was confirmed by Mu transposition in *E.coli* (data not shown), in a conjugation experiment [16,28].

For the expression of the MuA and MuB proteins in mammalian cells we cloned their coding regions in the pSuper-Catch-NLS vector (Fig. 1A). *In vitro* transcription and translation of the constructs enabled by the T7 promoter demonstrated the presence of products of the expected size that were recognized by the FLAG antibody. (Fig. 2). Besides some minor products, that were also obtained from the empty vector (pSuperCatch-NLS; lane2), pSC-NLS-MuA and pSC-NLS-MuB encode proteins of ~80 (lane 3) and 40 kDa (lane 4), respectively. In lysates of 911 cells (lane 1), stably expressing the modified MuA and MuB genes, products of identical sizes can be seen. These products were absent in the cell lysate of the unmodified parental 911 cells (data not shown).
The nuclear localization of both fusion proteins, a prerequisite for transposition

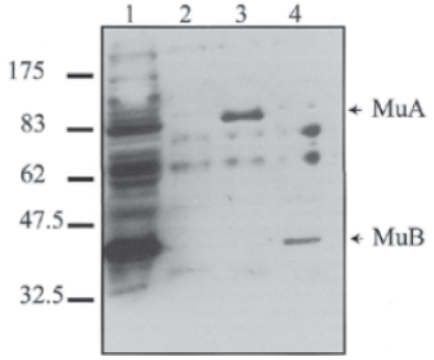

Figure 2. *Western analysis of in vitro and in vivo expression of pSC-NLS-MuA and pSC-NLS-MuB. Lane 1, total cell lysate (40 µg protein) of a 911-derived cell line, stably transfected with pSC-NLS-MuA and pSC-NLS-MuB; lanes 2, 3 and 4, the in vitro transcription translation products of pSuperCatch-NLS, pSC-NLS-MuA and pSC-NLS-MuB, respectively. Arrows, location of the fusion proteins, MuA and MuB.*

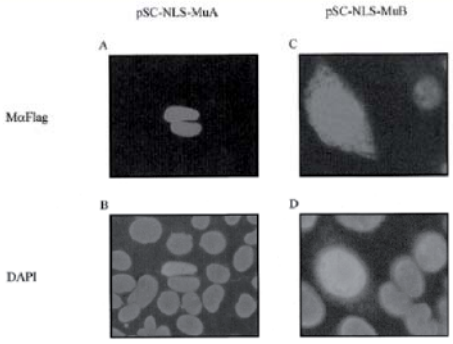

Figure 3. *Immunofluorescence of 911 cells transiently transfected with pSCNLS-MuA or pSC-NLS-MuB. (***A** *and* **C***) Visualize the presence of the MuA- and MuB-fusions, respectively, after staining with MαFlag and GαMFitc antibodies.(***B** *and* **D***) Nuclei of the same cells by DAPI staining.*

in mammalian cells, was evaluated by immunofluorescence. After a transient transfection of 911 cells with either pSC-NLS-MuA or pSC-NLS-MuB, it was clearly shown that both fusion proteins located predominantly in the nucleus (Fig. 3).

Functional analysis of the modified Mu proteins in *E.coli*

To establish the functionality of the MuA and MuB proteins fused to the NLS and the FLAG epitope, they were analyzed in a complementation assay in *E.coli*. In this assay pSC-NLS-MuA and pSC-NLS-MuB were used to complement the deficiency in Mu phages carrying MuA amber and MuB amber mutations, respectively. The complementation was determined by phage replication on KMBL 1001, transformed or not transformed with pSC-NLS-MuA or pSC-NLS-MuB.

The expression of MuA and MuB in KMBL 1001 transformants was confirmed by western analysis (data not shown). Furthermore, the replication of the MuA or MuB amber phages on KMBL 1164, containing the amber suppressor Sup E was evaluated (Fig. 4). As expected, the MuA amber and MuB amber phages replicated normally in KMBL 1164. The titre of the harvests, as determined on KMBL1164 was 2.1×10^6 and 3.7×10^7 p.f.u./ml, respectively. As a control, the same harvests were also titrated on KMBL 1001. Due to the lack of Sup E, no plaques were formed ($<10^2$ p.f.u./ml), confirming the presence of the amber mutations. If, however the MuA *am* and MuB *am* phages were grown on KMBL 1001, which was transformed with pSC-NLS-MuA and pSC-NLS-MuB, respectively, efficient propagation of the phages was observed, as determined by titration of each harvest on KMBL 1164. (Fig. 4). When these phage

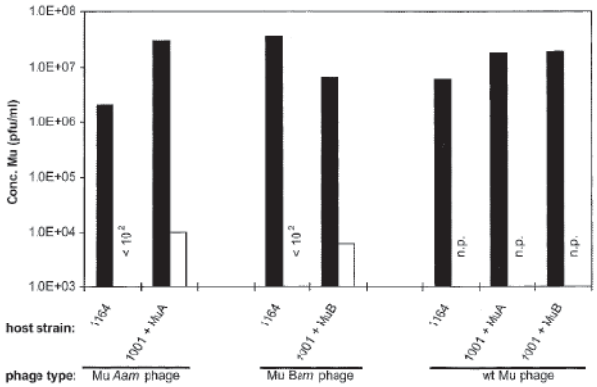

Figure 4. *Complementation of MuA amber and MuB amber phages by pSC-NLS-MuA and pSC-NLS-MuB, respectively. Indicated bacteriophage types were replicated on KMBL 1164 (1164), KMBL 1001 + pSC-NLS-MuA (1001 + MuA) or KMBL 1001 + pSC-NLS-MuB (1001 + MuB). The resulting harvests were titrated on KMBL 1164 (black bars) or on KMBL 1001 (white bars). n.p., not performed; KMBL 1164, [SupE$^-$], E.coli strain with suppressor of amber mutation; KMBL 1001, [SupE$^-$], E.coli without suppressor of amber mutation.*

titres were assayed on KMBL 1001, a severe reduction in titre was seen again. However, the titres obtained (1×10^4 p.f.u./ml for MuA *am* phages and 6×10^3 p.f.u./ml for MuB *am*) were higher than expected. This can be explained by homologous recombination between the wild-type MuA and MuB genes on the plasmids, and the mutated genes in the phages.

The obtained titres, as determined on KMBL 1164, were in accordance with those from wild-type bacteriophage-Mu harvests. Replication of wild-type Mu on KMBL 1164, KMBL 1001 transformed with pSC-NLS-MuA or KMBL 1001 transformed with pSC-NLS-MuB resulted in titres of 5.9×10^6, 1.8×10^7 and 1.9×10^7 p.f.u./ml, respectively. From these data we conclude that the N-terminal extensions of the MuA and MuB genes do not impede their functions.

Transposition in mammalian cells

To test the feasibility of the use of the Mu transposition machinery in mammalian cells, we studied the number of colonies obtained after transfer of the donor construct, pmMuHyg-GFP, with or without the expression cassettes for MuA and MuB. As can be seen in Table 1, the introduction of MuA increased the number of Hyg^R colonies with not more than 17%. The combined introduction of MuA and MuB, however, gave rise to an 81% increase in colony number. The positive effect of MuA on the number of stable transfectants was also obtained in transfections with other donor constructs (data not shown). Since the GFP marker of pmMuHyg-GFP was located outside the miniMu transposon, the absence of GFP-expression in the Hyg^R colonies could be an indication for bona fide Mu transposition via simple insertion. Although co-transfection of pmMuHyg-GFP and pSC-NLS-MuA in the absence or the presence of pSC-NLS-MuB resulted in 2 and 11% GFP-negative colonies, respectively, it only partially explained the raise in Hyg^R colonies.

To establish the contribution of co-integration to the observed raise in Hyg^R colonies, we linearized the donor construct in the subsequent co-transfection series. In this way, co-integration will lead to chromosomal breakage, leaving simple insertion as the only option for the production of a viable Mu-induced stable transfectant. The effect of the linearized donor template in a series of 1–0.1 µg on the number of Hyg^R colonies is shown in Table 2. In all cases the combined introduction of pSC-NLS-MuA and pSC-NLS-MuB resulted in more Hyg^R colonies than the control in which pSC-NLS-MuA was omitted. This effect was not seen when the donor construct was replaced by pCMVNeo. Furthermore, we found a pronounced effect of the linearization of the donor construct. The elimination of co-integration reduced the formation of Hyg^R colonies from 181 (Table 1) to 124%, in relation to their control without MuA. This reduction could be abolished when the amount of donor template was optimized. Compared to the number of colonies obtained in co-transfections without pSC-NLS-MuA, we found an increase to 124% for 1 µg template, 208% for 0.3 µg template and 266% for 0.1 µg template in combination with pSC-NLS-MuA.

Since direct evidence for Mu-mediated transposition can only be found at the molecular level, we analyzed the chromosomal DNA of G418-resistant colonies that were obtained after co-transfection of pSC-NLS-MuB and pSC-NLS-MuA with the donor construct pmMuNeo (Fig. 1B). From an experiment in 911 cells, 20 G418-resistant monoclonal cell lines were established. In parallel, from an experiment in U2OS cells we established 23 G418-resistant monoclonal cell lines and one polyclonal. The miniMu transposon of pmMuNeo contains both a eukaryotic and a prokaryotic expression cassette for the Neo^R marker in combination with a pBR322-derived origin-of-replication. This facilitated recovery and molecular cloning of the DNA flanking the inserted

Table 1. *In vivo analysis of the Mu transposition machinery by co-transfection into 911 cells*

Co-transfection				HygR colonies			
				Total		GFP–	
pmMuHyg-GFP	pSC-NLS-MuA	pSC-NLS-MuB	ssDNA	Number	%	Number	% of total
1 µg	-	2 µg	7 µg	72	100	0	0
1 µg	2 µg	-	7 µg	84	117	2	2
1 µg	2 µg	2 µg	5 µg	130	181	14	11

The effect of the indicated co-transfections of the circular donor construct pmMuHyg-GFP with pSC-NLS-MuA and/or pSC-NLS-MuB was measured by the amount of resulting HygR colonies. The GFP status was used here as indication for possible Mu-induced integration, where the absence of GFP expression (GFP–) suggested Mu transposition via a simple insertion of the miniMu transposon.

Table 2. *Effect of linearization and quantity of the donor construct, pmMuHyg, on the formation of Hyg^R colonies by the Mu transposition machinery*

Co-transfection					HygR colonies		
pmMuHyg (lin.)	pCMVNeo (lin.)	pSC-NLS-MuA	pSC-NLS-MuB	ssDNA	Exp. 1.	Exp. 2.	Average MuA effect
1 µg	-	-	2 µg	7 µg	152	50	100%
1 µg	-	2 µg	2 µg	5 µg	174	67	124%
0.3 µg	-	-	2 µg	7.7 µg	82	17	100%
0.3 µg	-	2 µg	2 µg	5.7 µg	119	46	208%
0.1 µg	-	-	2 µg	7.9 µg	14	19	100%
0.1 µg	-	2 µg	2 µg	5.9 µg	48	36	266%
-	0.1 µg	-	2 µg	7.9 µg	98	125	100%
-	0.1 µg	2 µg	2 µg	5.9 µg	71	89	72%

Indicated amounts of linearized donor construct were co-transfected with pSC-NLS-MuA and/or pSC-NLS-MuB into 911 cells and the resulting number of Hyg^R colonies was counted. The effect of MuA on the efficiency to form stable transfectants is shown in the last column. The results obtained without pSC-NLS-MuA are regarded as 100%. All transfections were performed with 10 µg DNA. Lin, linearized.

transgenes. To this end, chromosomal DNA of the monoclonal and polyclonal cell lines was isolated, digested with *Spe*I (to clone the left-hand border) or *Bam*HI (to clone the right-hand border), re-circularized by ligation and used to transform *E.coli*. The Kan^R transformants were subsequently tested for Amp resistance, restriction analysis and PCR analysis. In total we analyzed 37 right-hand borders and 143 left-hand borders (Table 3). In none of these we found a junction at the att sites, which would have been used in case of a bona fide Mu integration. To verify the efficiency of the procedure, we sequenced some of the junctions and confirmed that the junction between donor and target DNA was not at the att sites. Thus we could not find the 5 bp duplication of host DNA, which is characteristic for a Mu-insertion event.

DISCUSSION

A precisely defined integration as performed by transposons holds great promise for gene therapy. Especially, since in mice the integration efficiency of the Tc1-like transposase, Sleeping Beauty, was shown to equal the efficiency of lentivirus and rAAV [4]. This encouraged us to evaluate the transposition mechanism of bacteriophage Mu, one of the best studied and most efficient transposition machineries, for its applicability in mammalian cells. The transfer of the Mu system from prokaryotic

cells to mammalian cells required several adaptations, such as the addition of a Flag-epitope and an NLS. These extensions did not hamper their original functionality, as was confirmed by a complementation assay in *E.coli*. The expression of MuA and MuB from their mammalian expression vectors was confirmed in *in vitro* and *in vivo* analyses, which revealed their correct size and translocation to the nucleus.

In vivo analysis of the functionality of the Mu transposition machinery was performed by transfection of 911 or U2OS cells. Although less efficiently integrated than the circular donor templates, the linear donor construct in combination with MuA and MuB increased the number of stable transfectants to maximally 266%. In contrast to the effect of MuA and MuB, the use of ClpX, an *E.coli* protein which facilitates the disassembly of the transpososome [29], was less favourable in co-transfection. Although the positive effect of MuA and MuB was still observed, presence of ClpX showed a dramatic reduction in Hyg^R colonies, which suggests a cytotoxic effect in the tested mammalian cells (data not shown). Molecular cloning of 43 monoclonal and one polyclonal integrants and subsequent characterization of the junction fragments failed to provide evidence for bona fide Mu-mediated transposition. This suggests that the transfectants originate from non-homologous recombination, a process that is apparently positively affected by the presence of the MuA and MuB proteins in combination with a donor construct. It is tempting to speculate that binding of the nucleus-targeted Mu proteins to the att sites facilitates the transport of the donor construct into the nucleus [30,31].

The Mu-defined transposition was considered to be an ordered stepwise mechanism, until recently when new transpososome intermediates were described, which suggested alternative reaction pathways [13,32]. This implies that, even without the transposase's functionality of strand cleavage and joining, the DNA-binding capacity of MuA and MuB can induce the formation of a so-called type-0 target capture (TC-0) complex. This complex consists of MuA and MuB proteins, which bring the donor and target DNA in close proximity. Since in our situation the cleavage/joining reactions are not observed, this might favor non-homologous recombination. This hypothesis is supported by the observed relative increase in Hyg^R colonies, which was dependent on which Mu proteins were provided. When the co-transfection consisted of the donor construct and the MuA and MuB expression vectors, we observed an increase to 181%. This was reduced to 117%, when MuB was omitted from the co-transfection (Table 1). Moreover, when the donor construct was omitted, the positive effect of MuA and MuB on the amount of stable transfectants was completely lost (Table 2).

Obviously, the processes which underlie the increase in stable transfectants and the absence of detected Mu-specific integrations need to be elucidated further. It is however questionable whether an active Mu transposase can be established in mammalian cells and whether this will increase the efficiency of

Table 3. *Analysis of the chromosomal DNA from stable transfectants established after co-transfection of the indicated mammalian cell line with pSC-NLS-MuA, pSC-NLS-MuB and pmMuNeo*

Transfection		Analysis L att border		Analysis R att border	
Cell line	Number of Neo^R cell lines	mc/pc	Kan^R clones	mc/pc	Kan^R clones
911	20 mc	18	61	2	20
U2OS	23 mc	23	77	6	17
U2OS	1 pc	1	5	–	–

The Kan^R clones obtained from Mu-originated transfectants, would harbor the Neo^R marker in combination with the L att or R att domain and the flanking chromosomal DNA. mc, monoclonal cell lines; pc, polyclonal cell line.

integration to such extent that it can compete with existing integration systems [4].

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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

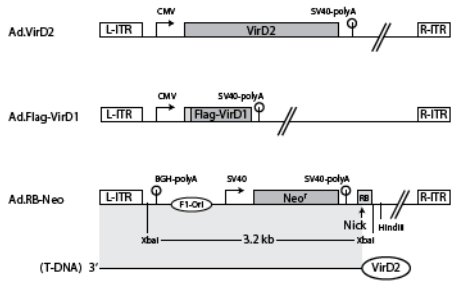

Figure 1. *Schematic presentation of the recombinant Adenoviral constructs. Both Ad.Flag-VirD1 and Ad.VirD2 contain a Cytomegalovirus Immediate Early promoter (CMV) driven expression cassette and the SV40 poly-adenylation signal. The VirD1 gene is fused with a Flag-epitope at the N-terminus. The Ad.RB-Neo 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)

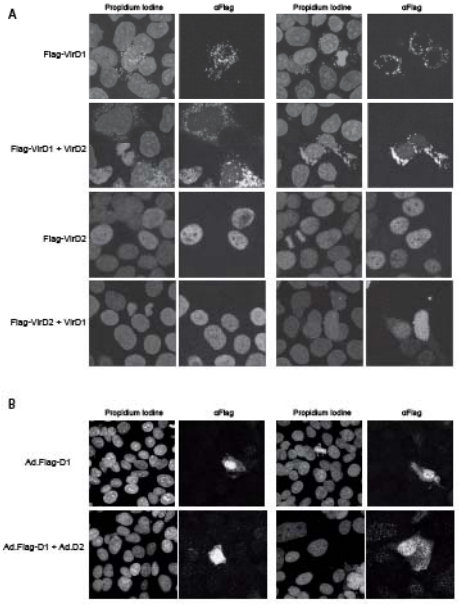

Figure 2. *Immune fluorescence assays of transfected and infected U2OS cells. U2OS cells were transfected with expression vectors for Flag-VirD1, Flag-VirD2 or a combination with non tagged constructs as indicated (A). The localizations were compared to U2OS cells infected with Ad.Flag-VirD1 or co-infected 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.*

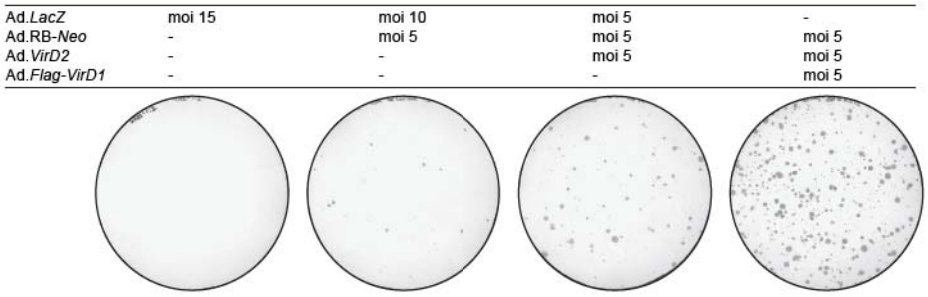

Figure 3. *Formation of G418 resistant colonies. To determine integration efficiencies, U2OS cells were infected with Ad.RB-Neo and Ad.LacZ or Ad.RB-Neo, Ad.VirD2 and AdLacZ or Ad.RB-Neo, Ad.VirD2 and Ad.Flag-VirD1. In each case and MOI of 5 was used for the Ad.RB-Neo, Ad.VirD2, and Ad.Flag-VirD1 viruses. 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

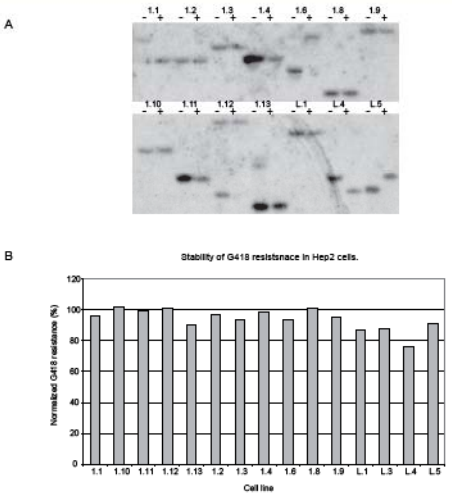

Figure 4. *Stability of G418-resistant colonies. A) Hep2 monoclonal cell lines have been isolated twenty days post infection. These monoclonal cell lines were cultured for 65 days in the presence or absence of 400 µg/ml G418 and genomic DNA was isolated. The genomic DNA was digested with HindIII, size 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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CHAPTER 7

Insertion vectors for gene therapy

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Chapter 7

Insertion vectors for gene therapy

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It goes without saying that efficient gene transfer is essential to gene therapy. Many different gene-transfer systems are being developed for numerous applications. Each of the envisaged applications has its own particular requirements and therefore a generally applicable, 'ideal vector' does not exist. Vector systems are usually divided into those where the vector inserts itself into the host genome and those with vectors that remain extra-chromosomal. Integrated vectors are the system of choice if persistent expression is required. However, their insertion results in an alteration of the chromosomal DNA and therefore may lead to insertional mutagenesis. Although the risk of insertional mutagenesis is a recognized disadvantage, many gene therapy protocols embrace the use of integrating vectors. Apparently, the small possibility of adverse effects of insertion mutagenesis is not seen as a major obstacle for the use of integrating vectors for clinical gene therapy.

Retroviruses are the archetype for vectors that integrate themselves into the host-cell genome. The murine retroviruses from which they are derived are notorious for their propensity to induce tumors in their natural hosts (hence their classification in the subfamily Oncovirinae). Once integrated, the provirus can perturb the expression of nearby cellular genes. In some instances, such a mutation can constitute a discrete step in the multistep process that eventually leads to cellular transformation. The unequalled efficiency with which retroviral vectors integrate their genome into the host-cell chromosomal DNA has made them the system of choice for many gene therapy applications. They have been used extensively in human gene therapy research. In more than 175 clinical trials, over 1600 patients have received retrovirus vectors. So far, not a single case has been reported in which adverse events have been attributed to insertional mutagenesis.

As murine retroviruses need both nuclear membrane degradation and active DNA synthesis in order to integrate,[1] their use is limited to applications in which mitotically active cells are to be modified. The relatively short half-life of the pre-integration complex limits the window during which infection leads to provirus integration to only a few hours. In contrast to the murine retrovirus-derived vectors, lentivirus vectors are suitable for gene transfer into nonmitotic, quiescent cells, as their pre-integration complex can cross the nuclear membrane. Therefore, lentivirus vectors appear more suitable for *in vivo* gene transfer and major research efforts have been instigated to develop safe and effective vector systems. With the current systems, helper-free stocks of vectors which retain less than 5% of the original lentiviral genome can be generated.[2]

The adeno-associated virus (AAV) has also been used as a vector. AAV is a member of the Dependovirus group of the parvovirus family. This virus requires the presence of adenovirus or herpesvirus as helper, for productive infection. In the absence of helper virus, the viral genome can integrate into the cellular DNA. In the presence of the

AAV-derived *rep* protein, the viral genome integrates preferentially at one locus on human chromosome 19. Upon subsequent infection with the herpes or adenovirus helpers, AAV can be activated. Whereas the integration of wild-type AAV has been documented in detail, the situation with AAV vectors is less clear. Although evidence suggests that at least a fraction of the AAV vectors persist as integrated copies, significant amounts of vector DNA appear to be maintained as large episomal concatemers.[3]

At present, the above-mentioned integrating vectors are the most widely used, but there are also ongoing efforts to generate hybrid vectors, which would be able to integrate into the genome of nonmitotic cells. Several attempts have been made to provide nonintegrating vectors (eg adenovirus vectors), which have the capacity to transduce nonmitotic cells, with integration mechanisms in order to insert the transgene into the host-cell genome. This would achieve the best of both worlds. The adenovirus–retrovirus chimeric vectors are an example of this new approach. This system consists of adenovirus vectors, which carry the *gag*, *pol* and *env* genes required for packaging the retroviral vector genomes, and an adenovirus vector that contains a recombinant provirus. When cells are co-infected with the above vectors, the transduced cells start to shed progeny retroviral vectors which can infect and stably modify the neighboring cells. In this way, the high titer of adenoviruses is combined with the integration capacity of retroviruses. Note that with this strategy only mitotically active cells can be stably modified.

A strategy that should be applicable to nonmitotic cells too, has recently been reported and involves the use of an AAV–adenovirus hybrid.[4] In this case, one adenovirus contains a transgene flanked by sequences of the AAV inverted terminal repeat and the other carries the AAV1derived *Rep78* gene. When cells are co-infected with this system, up to 35% of the clones that express the transferred resistance marker contain the transgene integrated near the hotspot of integration on chromosome 19.[4] Intriguingly, in the latter study, the frequency of integration appears to be independent of the presence of the *Rep*-expressing vector, but in the absence of *Rep78* the preferential integration into chromosome 19 is lost. A similar concept inspired the herpes simplex virus type I(HSV)/AAV hybrid vectors.[5] With such vectors, transgene expression was prolonged as compared with traditional HSV-I amplicon vectors. However, in the latter study no evidence was presented confirming the integration of the hybrid vector-derived AAV cassette.

Similarly, integration or recombination systems of other origins have been exploited for integration of trans-genes into the host cell genome. Such systems include various members of the transposable elements from the Tc1/mariner transposon family.[6-8] In all these studies, pre-defined parts of the vector will integrate into the host cell genome. Consequently, such hybrid vectors have the risk of insertional mutagenesis in common with the more conventional integrating vectors.

The absence of integration is often used as a safety argument to promote the use of non-integrating vectors, such as adenovirus-derived vectors or plasmid vectors. This argument should be used with caution. Adenovirus DNA can recombine with chromosomal DNA, and as a result, vector sequences can become integrated into the host-cell genome. Recently, Harui and co-workers[9] demonstrated that the frequency of adenovirus integration into chromosomal DNA was around 10^{-3}–10^{-5} events per cell. However, these frequencies have been determined in established cell lines, and it is not clear whether it is justified to extrapolate these frequencies to the *in vivo* situation and to estimate the risk. It would be very interesting to know the frequency with which the adenovirus vectors integrate

into the host-cell genome of diploid cells. In a study with *E1*-containing, but replication-defective adenovirus vectors in cultures of rat diploid kidney cells, Fallaux *et al*[10] reported the frequent occurrence of transformed foci. This indicates that in diploid cells too, the *E1* region can become integrated into the host-cell genome as a result of illegitimate recombination, although the E1 proteins might influence the frequency of integration.

In rodents and in hamsters, adenoviruses can also become integrated into the host-cell genome, as is evidenced by the occurrence of *E1*-containing tumors upon injection of, for example, subgroup-A adenoviruses. If one assumes the integration frequencies *in vivo* to be equivalent to those observed in cell lines, then the vector amounts used for *in vivo* gene therapy (up to 10^{13} vector-particles per dose) would imply that a significant number of cells (even millions) may acquire genome-integrated vector fragments. Thus, it is not strictly accurate to categorize adenovirus vectors as being non-integrating, when considering the risk of insertional mutagenesis. It should be noted that the insertion of vector sequences is not provoked by the adenovirus but, rather, is the result of illegitimate recombination. It seems, therefore, justified to assume that the use of other vector systems that introduce naked DNA (eg plasmids) into nuclei will also be accompanied by the integration of vector sequences into the host-cell genome.

This being the case, the use of 'non-integrating' vectors will be associated with a small but finite risk of insertional mutagenesis and perturbation of (proto-)oncogenes and tumor-suppressor genes, similar to the situation with retroviruses. However annoying this may seem at first sight, it should be seen in its proper perspective. In healthy human cells, chromosomal DNA is definitely not static and subject to mutation and recombination. Also germ line and somatic cells are modified, at a fairly high frequency, by transposable elements.[11] In all, it will be extremely difficult to estimate and assess the increased risk (if any) of gene therapy for insertional mutagenesis. As long as the potential benefit outweighs the risk one should not hesitate to choose gene therapy for the treatment of severe disorders, but it is essential to ensure a proper follow-up of all patients who participate in clinical trials. In addition, great care should be taken to provide balanced views of the risks associated with the gene-transfer techniques and to abstain from sweeping generalizations. If given proper counseling, the people involved can make their judgments based on their personal perception of the risk and benefits. We should realize that we are making such judgments every day. Don't most of us expose ourselves to sunlight to acquire a tan, at the cost of an increased risk of skin cancer due to UV-induced mutagenesis?[12]

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CHAPTER 8

General discussion

Discussion

In this thesis experiments are described that aim at vector improvements resulting in the generation of safer and more efficient Ad vectors. The first part of the thesis (chapters 2, 3 and 4) adresses the generation of mobilization-resistant Ads while in the second part (chapters 5, 6 and 7) we study the applicability of two integration systems to improve long-term gene expression and comment on the safety of integrating vectors.

Mobilization. The currently used third generation rAds are replication deficient due to the deletion of *E1*. These vectors are considered to be safe for use in human gene therapy trials since they are not able to replicate autonomously, thus preventing undesirable vector spread. However, in **chapter 2** we show that in cells, coinfected with a rHAdV-5 vector and *wt*HAdV-5, the wild-type virus is able to complement the *E1* deficiency and can mobilizes the rHAdV-5 vector. Furthermore, we show that not only the homologous wtAd vector mobilizes, but that all HAdV serotypes tested are able to mobilize the rAdV-5 vector. When used in patients, vector mobilization can result in uncontrolled spread of the rHAdV-5 vector. This can result in unexpected side effects, especially if the transgene has a small therapeutic window (e.g. where therapeutic dose should be high enough to get effect but overexpression will yield adverse effects). Examples are activators of the immune system, anti-tumor therapies using death receptors and the use of tumor selective promoters to drive the expression of suicide genes. The chance that *wt*Ads are located at the site of injection is the highest for sites where *wt*Ads replicate (e.g. eyes, lung and intestine). For all other tissues, the chance that HAdV-5 vectors can be mobilized by wtAds is relatively low for immune competent patients, unless the vector batch is contaminated with RCA or the tropism of the HAdV-5 vector has been genetically altered. To prevent vector mobilization by *wt*Ads, we aimed at the introduction of selectivity at the level of replication initiation.

Based on the fact that replication initiation sites have conserved cytidine residues in all HAdVs and the inability of HAdV-5 pTP to bind other nucleotides than dCMP *in vitro* [1], we mutated these nucleotides in the replication-initiation sites from dCMP to dGMP. This resulted in the inability of these mrAd plasmids to produce viral vectors when transfected in producer cell lines (**chapter 3**). To propagate these vectors, changes in the replication-initiation proteins are required to allow them to initiate replication with dGMP. For that reason we looked at all known Ad ITRs and found that FAdV-1 isolate PHELPS contained guanosine residues at the replication-initiation sites while the closely related isolate OTE contained cytidine residues according to the human Ads. So, the PHELPS proteins involved in replication-initiation should be able to bind dGMP. Sequence differences in the replication proteins of OTE and PHELPS might explain the difference in the initiating nucleotide. Therefore, we determined the coding sequence of OTE pTP and Pol and compared it with the PHELPS pTP and Pol sequence. Although minor differences were found between the PHELPS and OTE replication proteins, these differences could not explain a changed specificity for the replication-initiation substrate. We postulated that FAdV-1 has a relaxed specificity, i.e. it can use both dGMP and dCMP as substrate for replication initiation. We confirmed the relaxed specificity with a mini-replicon assay. Both PHELPS and OTE were able to replicate a small linear DNA fragment containing ITRs that resemble either PHELPS or OTE.

Comparing the predicted secondary structure of pTP resulted in the hypothesis that the distance between helices flanking the nucleotide-binding site in pTP might be responsible for the relaxed specificity. Therefore, in **chapter 4** we exchanged the nucleotide-binding pocket of HAdV-5 pTP for the postulated nucleotide-binding pocked of PHELPS. The hybrid pTP (H2pTP) was stably expressed in 911 cells, generating cell line 911-*H2pTP*. Transfection of mrAdCMV*GFP* plasmids in 911 cells did not result in the formation of virus. Transfection of reverted mrAdCMV*GFP* plasmids in which the ITRs were deliberately changed back to *wt*HAdV-5 sequences, did result in the production of virus. This demonstrated that the backbone of the mrAdCMV*GFP* vector was intact and the block of virus production was the result of the changed ITRs in mrAdCMV*GFP*. Transfection of mrAdCMV*GFP* plasmid to 911-*H2pTP* cells resulted in virus production similar to rAdCMV*GFP* on 911 cells. From these experiments we concluded that over-expression of H2pTP did allow for the replication of mrAd vectors. When mrAdCMV*GFP* vectors that were isolated from 911-*H2pTP* were used to infect 911 cells, absence of virus spread was anticipated due to the absence of H2pTP. Unexpectedly, two-days post infection, a cytopathogenic effect (CPE) was apparent indicative for replicating virus, which was not caused by AdCMV*GFP* or *wt*Ad contamination. When mrAdCMV*GFP* virus propagated on 911 cells was isolated, the left ITR was sequenced and confirmed that the initial guanidine residues at position 1, 4, and 7 were converted to cytidine residues while the *Cla*I site was still present. Currently, the efficient reversal of guanidine to cytidine residues in mrAds limits the strategy to target replication for the prevention of mobilization.

This result was unexpected since transfection of mrAdCMV*GFP* to 911 cells never resulted in virus production, while transfection efficiencies were equal. What was more surprising was the reversal of the thymidine residue at nt 8 to alanine, suggesting either an event of homologous recombination with HAdV-5 like ITRs or replication initiation on 5'-CATCATCA templates. Since both ITRs in mrAds were confirmed to contain 5'-GATGATGTAT and no *wt*Ads were present, homologous recombination is unlikely. Next, we searched in the genomic sequence database for ITR like sequences containing at least part of the repeat region upstream of the pTP/Pol binding consensus. We found several sites that might act as replication-initiation sites. So far, it is not known whether the H2pTP or the residual genomic HAdV-5 pTP is responsible for this reversion. Similar experiments with Ads deleted for pTP are required to see if the replicative advantage of revertants is removed.

The most important potential application for these mrAds is their function as helper Ads for the production of gutless Ads. Gutless Ad vectors are almost completely stripped of viral sequences and therefore can harvest approximately 35 kb of exogenous DNA. Since these vectors rely on the delivery of viral sequences *in trans*, current production strategies depend on coinfection with a replication-competent Ad virus. Since both the gutless vector and helper vector replicate, methods to prevent or remove helper contamination are essential. The first strategy to prevent contaminating helper virus was to place LoxP sites around the packaging signal [2]. In the absence of Cre these helper vectors can be produced to normal titers. In a cell line expressing Cre, the packaging signal will be excised and only the gutless vector will be packaged. The excision of the packaging signal by Cre is dependent on Cre expression levels and the presence of LoxP sites. If the helper Ad has been produced on 293-*Cre* or 911-*Cre* cells, homology with Ad sequences in the cell line can result in homologous recombination near the packaging signal. This results in the loss of one or both loxP sites. The packaging signal

of these recombinants can not be excised, thus yielding helper Ad contamination. This problem can be easily overcome by using helper cell lines like PER.C6 [3] or N52.E6 [4], which are devoid of sequence homology with Ad vectors. Other groups have utilized Flp recombinase [5] with approximately equal efficiencies as Cre. Reversal of the packaging signal in gutless Ads reduced homologous recombination with the helper Ad, decreasing helper contamination to 0.02-0.1% [6].

Ideally, gutless vector batches should be free of helper virus since helper Ads are more prone to induce an immune response and can assist in the mobilization of gutless Ads. So, alternative methods are required to prevent helper vector contamination. The mrAds that we developed, depend for their replication on the presence of H2pTP. Without H2pTP, these vectors can deliver all genes *in trans* without *de novo* helper replication. This may aid in the generation of gutless Ads that are free of helper (mrAd) contamination.

The absence of viral replication might still have some limitation to the use as helper viruses. Ads lacking *E2A* are deficient for Ad replication and do not switch to late-gene expression. One reason might be that viral replication is required to accumulate enough template for efficient late-gene expression. If this is the case, the use of mrAds for the production of gutless Ads needs further improvement. One option is to use the Cre/Lox system to circularize the Ad genome, which is then able to replicate when combined with a SV40 origin and SV40 largeT.

One other possibility is that *E2A* has specific functions in regulating the major late promoter as was suggested by Chang and colleagues who measured the effect of DBP on MLP activity [7]. The enhancement of MLP activity is at least partially caused by enhanced transcription-factor binding due to DBP induced DNA bending [8]. The observation that pTP and TP localize to distinct nuclear regions can be a third problem. Since *de novo* replicated (pTP-containing) templates are targeted to genomic regions specific for viral gene expression, replication-deficient (TP containing) may be excluded from the genomic regions specific for transcription. It would be interesting to see if this problem occurs and if pTP mutated at the p23 protease cleavage sites can improve the transcription activity of these templates.

Integration. The longevity of transgene expression did increase considerably with the use of gutless Ads. However, for obtaining persistent transgene expression, transgene integration may be required as well. Especially if the target cell is dividing. To obtain Ads that can integrate their transgene casette in the host genome, we tried to adapt the bacteriophage Mu (**Chapter 5**) and *Agrobacterium tumefaciens* (**Chapter 6**) integration system. Initially, the bacteriophage Mu was chosen for its well-known integration mechanism and high integration efficiency in *E.coli*. The minimal components required for transposition are known and depended on the phage MuA and MuB proteins. Extracts from *E.coli* could enhance this reaction, indicating that accessory proteins also play a role in this reaction. The MuA and MuB-coding sequences were cloned in eukaryotic expression plasmids and the integration system was tested in 911 cells. The increase of integration when *MuA* and *MuB* were cotransfected with circular miniMu plasmid was 1.8 to 2.6 fold over background integration with linear miniMu templates. However, when stable integrants were sequenced to determine the genomic sequences flanking the integration cassette, no junctions were found in close proximity to the attachment sites. Alternative integration mechanisms must have been enhanced by MuA and MuB proteins. The integration efficiency of the Mu system in its current form is too low to be used with Adenoviral vectors. So far, the Mu integration system has successfully been applied in *in vitro*

recombination reactions [9] but no additional reports of the use of Mu in mammalian cells have been published so far.

In **chapter 7**, the *A.tum.* integration system was studied as an alternative mechanism for use in rAd vectors. The *A.tum.* soil bacterium generates single- stranded transferred DNA (T-DNA) molecules. These T-DNAs are injected in the plant cytosol and are subsequently transported into the host nucleus where they integrate. Since this integration normally takes place in plant cells where it is dependent on host factors, the *A.tum.* system might be more compatible with human cells than the Mu system. The minimal *A.tum.* requirements for T-DNA generation and integration in plant are the proteins VirD1 and VirD2. Expression of VirD1 and VirD2 in plant was shown to be sufficient to generate and integrate T-DNA in plant cells. Therefore we cloned the VirD1 and VirD2 coding sequences in Ads together with an Ad containing the integration cassette consisting of a neomycin resistance (Neo^R) cassette and a Right Border (RB) sequence. Coinfection of AdCMV*Flag-VirD1*, AdCMV*VirD2* and Ad.RB-*Neo* should result in the generation of T-DNAs in the human cells. Injection of synthetic T-DNA molecules has been shown to be able to integrate in HeLa cells [10]. When human cell lines were infected with AdCMV*Flag-VirD1*, AdCMV*VirD2* and Ad.RB-*Neo*, a 60-fold increase in neomycin-resistant colonies was detected compared to Ad.RB-*Neo* infected cells alone. Infection with Ad.*VirD2* and Ad.RB-*Neo* increased the integration efficiency only 12-fold. So addition of both AdCMV*Flag-VirD1* and Ad.*VirD2* did increase the integration efficiency of the Neo^R cassette. To show that the Neo^R cassette was stably integrated, we cultured monoclonal cell lines in the presence and absence of selection drug G418 for over 2 months. Subsequently, cells were again sunjected to and the number of colonies formed was compared to the number of colonies formed with cell that consistently received G418 selection. The number of G418 resistant colonies was similar in both cases, indicating that lack of G418 selection did not result in loss or silencing of the Neo^R cassette. Also, chromosomal DNA isolated from monoclonal cell lines cultured with or without G418 were analyzed for instability of the integrated Neo^R cassette, which were shown to be stable. Thus the integration enhanced by VirD1 and VirD2 appeared stable and efficient. However, when we analyzed the genomic DNA of the monoclonal cell lines for *A.tum.* specific integration or spontaneous integration, we could not find any proof for *A.tum.*-specific integration. When the Neo^R cassette would have been integrated according to the *A.tum.* integration system, an *Xba*I site, which was located just outside of the integration cassette, would have been lost. In all our cell lines, this site was still present suggesting random integration of a fragment encoding the T-DNA including some additional adenovirus DNA.

These studies showed that from both systems tested, the *A.tum.* integration system has the highest efficiency. The main drawback of the results we have obtained with both systems is the lack of integration according to their established mechanisms. This resulted in the presence of additional viral sequences of unknown size. The integration of the viral sequences can have unpredictable effects on the stability and gene expression of both the integration cassette as well as flanking genomic sequences. It is important to determine the flanking sequences from the *A.tum.* experiments. If pseudo attachment sites appear to be present, it might be possible to remove these sites from the viral genome. Since the VirD1/VirD2 complex needs supercoiled DNA to nick the bottom strand, the position of local supercoiling and thus the position of the borders in the viral genome might affect the nicking efficiency as well. Altogether, the unexpected results with both integration systems makes them

currently not suitable as reliable mechanisms for the stable integration of exogenous DNA in the genome of human cells.

So far, two other groups have reported about the use of the *A.tum.* integration system and mammalian cells. The first report appeared in 1998 where Relic and coworkers demonstrated the subcellular localization of VirD1 and VirD2 in mammalian cells [11]. Nuclear localization of VirD1 was dependent on the coexpression of VirD2. The absence of nuclear entry of VirD1 was unexpected since the size of VirD1 allows diffusion through the nuclear membrane. Our localization analysis of VirD fused to a N-terminal Flag epitope demonstrated that VirD1 is indeed both cytosolic and nuclear expressed. Posibly, the fusion of GFP to VirD1, as described previously in Relic's paper might have altered the localization of VirD1. In fact, Ziemienowicz and coworkers showed that *in vitro* generated T-DNA molecules once injected in the cytosol of HeLa cells could translocate to the nucleus [12]. Integration of these complexes were not shown until 2004 when Pelczar and coworkers demonstrated that these *in vitro* generated complexes allowed for precise and single copy integration in HeLa cells [10]. In 2001, Kunik and coworkers studied the transfer of T-DNA from *A.tum.* to HeLa cells by coculture [13]. We have tried to replicate those experiments by transferring a T-DNA expression a G418- resistance gene. Like the authors, we did observe the aggregation of *A.tum.* upon coculturing, but we were have not been able to establish G418-resistant colonies from these experiments.

With the development of lentiviral vectors, the need for persisting adenoviral vectors has been lowered since. In contrast to other onco-retroviral vectors, Lentiviral vectors are capable of integrating in non-dividing cells. With the ability to pseudotype these vectors with VSV-G envelop protein, host-range limitations are also solved although specific targeting is still not possible. And with the recent safety developments for this vector system, the four times smaller packaging capacity of lentiviruses might be the major remaining limitation. Still, efficient integration systems for Ads can have major advantages. Though the integration system of Lentiviral vectors is efficient, the integration itself is random and not easily adjustable. The possibility to have site-specific integration is a major advantage over random integration. Eventually, targeted integration will be the ultimate goal for long-term gene therapy.

Though targeted integration is not possible yet, a major improvement over random integration is site-specific integration. Current systems with such potential are the Phage φC31, which has prevalence for specific sites in the human genome, and AAV which, in the presence of Rep, can integrate at defined region on chromosome 19 in human cells. The limited packaging capacity of AAV vectors does make it an unsuitable vector for a lot of applications but the recent development of Ad/AAV hybrid vectors is very promising though integration efficiencies are currently not known [14].

If, in the future, integration systems can be developed that make use of homologous recombination, gene correction instead of gene addition might become a possibility. The correction of affected genes within its natural locus will prevent all kinds of secondary effect that occur when genes are added randomly to the genome. In addition, these vectors will be very powerful tools to study the gene function.

With the increased efficiency that is obtained with integrating vectors, there is also an increased safety risk. In **chapter 7**, we discuss the risks of vector integration and put these risks in perspective. Though the risk of insertional mutagenesis of retroviral vectors is well acknowledged, the fact that vectors lacking dedicated integration systems appear to integrate as well, albeit with low efficiency, is less appreciated. Since time of publication, three examples of adverse

effects due to insertional mutagenesis are known but the overall conclusion still remains positive. Despite the fact that the risk of insertional mutagenesis is finite, it is small concerning the number of trials without adverse effects. While the risk of insertional mutagenesis should never be neglected, it should also not be over dramatized and prevent further developments in this field. When using integrating vectors in the clinic, it is important that patients are well informed so a well balanced decision can be made were potential benefit is weighted against the risks.

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Samenvatting

Samenvatting

Dit proefschrift beschrijft experimenten die tot doel hebben het verbeteren van Adenovirale genoverdracht vectoren voor gentherapie. Met gentherapie wordt bedoeld het behandelen van patiënten door middel van DNA overdracht in somatische cellen van de patiënt. In de meeste gevallen zal dit DNA een gen zijn dat codeert voor een 'gezond' eiwit om zo de functie van een 'defect' eiwit over te nemen. Zo zijn er al jongetjes behandeld die een defect in het eiwit γc hebben waardoor bepaalde afweercellen niet meer kunnen functioneren. Deze jongens hebben een niet-normaal functionerend immuunsysteem en kunnen overlijden aan infecties die in gezonde personen niet eens zouden worden opgemerkt. Bij deze studies zijn de voorlopercellen van deze afweercellen behandeld met DNA dat codeert voor een gezond γc eiwit. Doordat deze voorlopercellen zich nu wel kunnen delen en het γc eiwit maken, kunnen ze uitgroeien tot de afweercellen die de patiëntjes missen. De behandeling van deze patiëntjes resulteerde in eerste instantie tot een volledig herstel van het immuunsysteem. Echter, na drie jaar bleek dat bij sommige jongens de herstelde afweercellen sneller deelden dan normaal, het geen uiteindelijk resulteerde in leukemie. Dit voorbeeld geeft aan dat gentherapie de potentie bezit om bepaalde erfelijke ziekten te behandelen, maar het geeft ook aan dat er nog veel moet worden geleerd over deze toepassing.

Bij gentherapie wordt het gezonde DNA in een cel gebracht zodat deze cel hiervan het gezonde eiwit kan maken. Nu zijn er verschillende manieren om dit DNA in de cel te brengen. De meest efficiënte manier is die met behulp van virussen. Organismen hebben geavanceerde afweersystemen ontwikkeld om infecties door deze virussen zo veel mogelijk te voorkomen. Virussen die zich aan deze afweer weten aan te passen zijn in staat om zich te verspreiden. Door dit selectieproces is er een grote variatie aan virussen ontstaan die, elk met een verschillende efficiëntie, in staat zijn hun erfelijke informatie over te dragen aan cellen. Normale virussen zullen vervolgens vermenigvuldigen om zich weer verder binnen de gastheer en naar andere organismen te verspreiden. Een van de virussen die in het gentherapie vakgebied wordt gebruikt is het Adenovirus. Dit virus, dat normaal een milde verkoudheid veroorzaakt, wordt gebruikt omdat het van nature nauwelijks schadelijk is in gezonde patiënten, een grote variëteit aan celtypen efficiënt kan infecteren en relatief grote DNA-fragmenten kan overdragen. Omdat het uiteraard niet is toegestaan om een ziekteverwekker bij patiënten toe te passen is het virus zo veranderd dat het alleen nog maar in het laboratorium vermenigvuldigd kan worden. Dit is gedaan door één gen te verwijderen (het *E1* gen) dat er normaal voor zorgt dat er virale eiwitten worden gemaakt en één gen dat niet meer nodig is doordat de productie ervan in het laboratorium plaatsvindt (het *E3* gen). Deze virussen zonder het *E1* en *E3* gen worden vectoren genoemd. Doordat de *E1* en *E3* genen uit het virus zijn verwijderd (Δ*E1*/Δ*E3*) ontstaat er ruimte om DNA aan het virus toe te voegen dat tot genezing van de patiënt moet leiden. Deze vector ziet er aan de buitenkant net zo uit als de ziekteverwekkende variant (wild type virus) en kan daardoor net zo efficiënt het DNA afleveren in cellen van patiënten. Echter, doordat een deel van het virale DNA is vervangen door therapeutisch DNA, kan de vector zich in de patiënt niet meer vermenigvuldigen maar, zorgt het er wel voor dat de geïnfecteerde cel het 'gezonde' eiwit gaat maken.

Nu is dit een sterk vereenvoudigde uiteenzetting en is in de afgelopen decennia gebleken dat het niet eenvoudig is om patiënten te genezen met behulp van gentherapie. Wel hebben alle tegenslagen er

toe geleid dat we meer zijn gaan begrijpen over de afweer tegen virussen, celdeling en welke beperkingen er zijn om een geïnfecteerde cel langdurig een 'gezond' eiwit te laten maken.
In dit proefschrift is gewerk om verbeteringen in de vector aan te brengen voor twee van deze problemen. Ten eerste het maken van nog veiliger en efficiëntere Adenovirale vectoren. Ten tweede, het verbeteren van langdurige gen-expressie door het therapeutische DNA te laten integreren in het genoom van de gastheer.
In hoofdstuk **2** van dit proefschrift laten we een belangrijke tekortkoming zien van de standaard *E1*/*E3*-gedeleteerde humane Adenovirus Type 5 (HAdV-5) vector. Deze Δ*E1*/Δ*E3* vector kan en mag, in het belang van de patiënt, niet meer autonoom repliceren. Wij hebben laten zien dat in aanwezigheid van een wild type (wt) virus het *E1*/*E3* defect in de vector wordt verholpen waardoor de vectoren weer kan repliceren. Dit wordt mobilisatie van de vector door het wt virus genoemd. Dit was verwacht voor wt HAdV-5 omdat de vector afgeleid is dit wt virus. Wat we niet hadden verwacht is dat alle geteste wt Adenovirussen in staat waren om HAdV-5 vectoren efficiënt te mobiliseren. Dit heeft gevolgen voor het gebruik van deze vectoren in de kliniek. Omdat het niet is toegestaan om vectoren aan patiënten toe te dienen die ongecontroleerd kunnen verspreiden ontstaat er een probleem als deze patiënten tegelijkertijd met wt Ads zijn geïnfecteerd.
Het probleem van mobilisatie treedt ook op bij een meer recente vectorontwikkeling. Hoewel een Δ*E1*/Δ*E3* vector niet meer autonoom repliceert vindt er toch op een laag niveau productie van virale eiwitten plaats. Dit is weliswaar onvoldoende om virale replicatie te krijgen maar voldoende om het immuunsysteem van de patiënt te activeren. Het gevolg is dat binnen korte tijd de cellen die nu het gezonde eiwit zijn gaan maken vernietigd worden door het immuunsysteem omdat deze de cel nu ziet als geïnfecteerde cel. Bij de nieuwste vectoren zijn niet alleen de *E1*/*E3* genen verwijderd maar al het virale DNA dat codeert voor eiwit. Deze zogenoemde 'gutless-vectoren' kunnen geen viraal eiwit maken en in de praktijk blijkt dan ook dat deze veel minder efficiënt door het immuunsysteem van de patiënt worden herkend. De cellen geïnfecteerd met de gutless-vector kunnen dus veel langer het gezonde eiwit maken. Omdat er meer viraal DNA is verwijderd kan er ook meer therapeutisch DNA gebruikt worden wat een bijkomend voordeel van de gutless-vector is. Dit is dus een belangrijke verbetering ten opzichte van de Δ*E1*/Δ*E3* vectoren. Om deze vectoren te maken is een tweede virus (helper-virus) nodig. Dit helper-virus zorgt ervoor dat alle virale eiwitten worden gemaakt maar mag zichzelf niet meer verspreiden. Om verspreiding te voorkomen wordt tijdens de productie van dit helper-virus het 'packaging-signaal' verwijderd. Het packaging-signaal is een stukje DNA dat door een leeg virusdeeltje wordt herkend en er voor zorgt dat het viraal DNA in het virus deeltje wordt opgenomen. Het helper-virus maakt dus lege virus deeltjes en zorgt ervoor dat het gutless-virus DNA wordt gerepliceerd waarna het in het lege virusdeeltje wordt ingebouwd. Het verwijderen van het packaging-signaal is echter onvolledig waardoor er onbedoeld een beetje helper-virus tussen het gutless - virus aanwezig zal zijn.
Het probleem van Δ*E1*/Δ*E3* mobilisatie door wt Ads en de contaminatie van gutless-Ads met helper-Ads is mogelijk te voorkomen door het replicatiemechanisme van Δ*E1*/Δ*E3* Ads en helper-Ads zo te veranderen dat ze niet meer kunnen worden herkend door wt Ads. Hiervoor hebben twee Kippenadenovirus isolaten met elkaar vergeleken (FAdV-1 OTE en FAdV-1 PHELPS). Dit staat beschreven in hoofdstuk **3**. De genomen van OTE en PHELPS lijken erg op elkaar, maar hebben toch een heel belangrijk verschil. Het genoom van PHELPS begint met een G-nucleotide terwijl OTE, net zo

als alle andere Adenovirussen, met een C-nucleotide begint. Dit is interessant omdat tijdens de replicatie van het virus het eerste nucleotide altijd wordt gebonden door een viraal eiwit, het 'pre-terminal protein' (pTP). Dit pTP dient tijdens de replicatie van het virus als startplaats. Hierbij wordt door het polymerase enzym (Pol) het eerste nucleotide van het virale genoom aan pTP gekoppeld. In het geval van OTE is dat een C, maar in het geval van PHELPS moet dat dus een G zijn. We hebben nu bepaald hoe de eiwitsequentie van PHELPS pTP zich vergelijkt met de eiwitsequentie van OTE pTP. Als daar immers grote verschillen in zitten, dan zou dat kunnen verklaren waarom de ene C's en de andere G's bindt. We hebben zowel in pTP als in Pol geen grote verschillen gevonden die het verschil tussen C en G binding kunnen verklaren. Het lijkt er op dat zowel OTE als PHELPS de replicatie kan starten met zowel C's als G's. Dit hebben we verder getest door in cellen verkleinde versies van het OTE genoom (miniOTE) te transfecteren en te co-infecteren met OTE. Dit miniOTE genoom hebben we in twee versies gemaakt, één die begint met C's en één die begint met G's. We zagen dat zowel miniOTE-C als miniOTE-G wordt gerepliceerd door het OTE virus. Dit zelfde hebben we gedaan met PHELPS. Ook PHELPS bleek in staat om zowel het minigenoom dat begint met een G als met minigenoom dat begint met een C te laten repliceren. Hiermee hebben we aangetoond dat zowel OTE als PHELPS in staat is om de replicatie te starten van FAdV-1 genomen die met C's of G's begint.

Dit lijkt heel anders te zijn dan voor humaan Adenovirus. Voor HAdV-5 heeft men al aangetoond dat in een reageerbuis virale eiwitten alleen genomen die beginnen met een C kan laten repliceren. Om te controleren of dat ook zo is in cellen hebben de uiteinden van Δ*E1*/Δ*E3* virus veranderd zodat ze niet meer met een C maar met een G beginnen. Dit staat beschreven in hoofdstuk **4**. Het pTP en Pol dat op dit virusgenoom ligt zou dan niet meer in staat moeten zijn om de replicatie van dit virus op te starten. Als controle hebben we een vergelijkbaar virus genomen dat wel met C's begint. Als we dit controlevirus-DNA transfecteren naar cellen waarmee Adenovirus geproduceerd wordt (911 cellen) dan zien we na een aantal dagen een duidelijke amplificatie van het virus. Het mutantvirus dat met G begint (we noemen dit mdAd-virus) transfecteert net zo goed naar 911-cellen, maar levert geen amplificatie van virus op. Hieruit blijkt dat in cellen de amplificatie van HAdV-5 heel specifiek is voor genomen die beginnen met C's. Om mdAd-virus te produceren hebben we veranderingen nodig in pTP die ervoor zorgen dat er een G gebonden kan worden. We hebben alle bekende pTP's, inclusief die van HAdV-5 en de door ons bepaalde sequenties van PHELPS en OTE (hoofdstuk **3**) met elkaar vergeleken en gekeken naar de voorspelde structuur rond het nucleotidebindend domein. Het blijkt dat het aminozuur dat de C of G bindt geflankeerd wordt door twee helices. De grootte van de ruimte tussen de helices verschilt tussen OTE en PHELPS enerzijds en het HAdV-5 virus anderzijds. In HAdV-5 pTP is deze ruimte kleiner dan bij PHELPS en OTE pTP. De grootte van deze ruimte zou dus wel eens belangrijk kunnen zijn voor de binding van C- of het grotere G-nucleotide. Vervolgens hebben we in het DNA van HAdV-5 pTP de kleiner ruimte tussen de helices uitgewisseld met de grotere ruimte van PHELPS pTP. Dit nieuwe eiwit hebben we H2pTP genoemd. De 911-cellijn hebben we zo veranderd dat deze nu het H2pTP eiwit maakt en hem 911-H2pTP genoemd. Als we nu mdAd DNA transfecteren naar deze 911-H2pTP cellijn, dan vind er na een aantal dagen wel amplificatie van mdAd virus plaats, terwijl dit in afwezigheid van H2pTP niet gebeurt. Door de uiteinden van HAdV-5 te veranderen naar G's kunnen we dus de amplificatie blokkeren. Het uitwisselen van het domein in pTP rond het nucleotidebindend domein zorgt ervoor

dat dit mdAd nu wel geamplificeerd kan worden.
Het mdAd-virus dat is geproduceerd in 911-H2pTP cellen zou niet meer mogen repliceren op 911 cellen omdat het H2pTP dat nodig is voor de replicatie van genomen met een G, afwezig is in 911-cellen. We hebben het mdAd-virus uit 911-H2pTP cellen geïsoleerd en geïnfecteerd op 911 cellen. In tegenstelling tot onze verwachting bleek het virus nu wel op 911 cellen te repliceren. Een mogelijke verklaring hiervoor zou kunnen zijn dat genen die erg op elkaar lijken, soms uitgewisseld worden (homologe recombinatie). Om uit te sluiten dat pTP uit het virus homoloog gerecombineerd heeft met H2pTP uit de 911-H2pTP cellijn, hebben de sequentie van pTP uit mdAd-virus bepaald. Dit bleek nog het normale HAdV-5 pTP te zijn en kan dus niet verklaren waarom het virus wel repliceert op 911 cellen. Vervolgens hebben we gekeken naar de uiteinden van het virus zelf. Deze zouden moeten beginnen met een G. We hebben gecontroleerd dat het uitgangsconstruct inderdaad begint met een G. Echter, het virus dat repliceert op 911 cellen blijkt weer met een C te beginnen. Dit terwijl er even verderop in het genoom een voor mdAd-unieke ClaI sequentie nog steeds aanwezig is. Dus, het virus genoom dat met een G begon tijdens de transfectie 911-H2pTP heeft tijdens de amplificatie op 911-H2pTP cellen of op 911 cellen zijn G's omgewisseld voor C's. Het is nog niet duidelijk waneer en hoe dit precies is gebeurd.

Het andere probleem waaraan is gewerkt is de kortstondige duur van expressie van het gezonde eiwit doordat de virus vector met het gen dat codeert voor dit eiwit weer verloren gaat in snel-delend weefsel. Als een cel wordt geïnfecteerd met een niet-integrerend vector dat het gen voor dit eiwit bezit, dan kan deze cel het 'gezonde' eiwit gaan maken. Als de cel zich nu gaat delen, zoals in het geval van de voorlopercellen die γc eiwit moeten maken, dan verliest één van de twee dochtercellen het virale DNA waardoor deze cel de nieuw verworven eigenschap weer heeft verloren. Dit probleem is op te lossen door het therapeutische DNA te laten integreren in het genoom van de patiënt. Doordat het geïntegreerde gen met de celdeling meedeelt zullen beide dochtercellen het gen behouden waarmee de expressie van het 'gezonde' eiwit wordt verhoogd en verlengd. Een aantal virussen heeft de eigenschap om te integreren maar het Adenovirus is een niet-integrerend virus en kan zijn DNA dus niet overdragen naar het genoom van de patiënt. In hoofdstuk **5** en **6** beschrijven we de resultaten die we hebben gekregen door twee verschillende integratie-mechanismen te bestuderen. In hoofdstuk **5** is gekeken of het integratie-mechanisme van bacteriofaag Mu in staat is om DNA te laten integreren in gekweekte humane cellen. Bacteriofaag Mu infecteert normaal *Escherichia coli* (*E.coli*) bacteriën en geen humane cellen. Het is bekend dat in een regeerbuis het integratiesysteem van Mu gewoon werkzaam is. Hierbij zijn twee Mu-eiwitten betrokken: MuA en MuB. MuA en MuB zijn samen in staat om DNA dat geflankeerd is door zogenaamde 'Attachment sites' te laten integreren op een willekeurige plaats in donor DNA. Normaal heeft bacteriofaag Mu daar wel *E.coli* eiwitten voor nodig. Omdat humane cellen deze *E.coli* eiwitten niet bevatten is het dus ook niet zeker dat het systeem werkt in humane cellen. Wel is bekend dat humane cellen een aantal eiwitten bevatten die sterk op deze *E.coli* eiwitten lijkt. We vermoedden dat deze eiwitten de functie van de *E.coli* eiwitten over kunnen nemen. Normaal zijn MuA en MuB werkzaam in bacteriën maar omdat ze nu werkzaam moeten zijn in de kern van humane cellen, hebben we een kern-localisatie-signaal aan MuA en MuB gefuseerd. Eiwitten die dit signaal bevatten worden naar de celkern getransporteerd waar zich het genomisch DNA bevind. Om uit te sluiten dat het kern-localisatie-signaal de werking van MuA en MuB verstoord, hebben

we in *E.coli* laten zien beide gemodificeerde eiwitten nog steeds functioneel zijn. Door humane cellen te transfecteren met MuA, MuB en het te integreren miniMu plasmide, konden we laten zien dat MuA en MuB de integratiefrequentie met meer dan 2,5x verhoogt. Nadat de plaats van integratie in een aantal van deze integratiecellijnen was bepaald bleek echter dat dit geen bonafide Mu integraties waren. We concluderen daarom dat MuA en MuB de integratie van miniMu verhoogt, maar doet dit niet volgens het Mu integratiemechanisme.

In hoofdstuk **6** bestuderen we het integratiemechanisme van *Agrobacterium tumefaciens* (*A.tum.*). Deze bacterie komt voor in de grond en infecteert planten waar de bacterie tumoren kan veroorzaken. Deze tumoren zijn een gevolg van de overdracht van een stuk DNA (T-DNA) vanuit de bacterie naar de plantencel. Dit T-DNA wordt naar de kern van de plantencel getransporteerd en integreert daar in het genoom. Bij celdeling wordt het genoom uit de plantencel gedupliceerd en verdeeld over de twee dochtercellen. Omdat het T-DNA is geïntegreerd zal deze ook over de twee dochtercellen worden verdeeld. Op het geïntegreerde T-DNA liggen genen die celdeling stimuleren. Omdat na celdeling de dochtercellen ook het T-DNA bevatten, zullen deze ook weer versneld delen. Uiteindelijk resulteert dit bij de plant in de vorming van een tumor. Genen op dit T-DNA zorgen er daarnaast voor dat de plantencellen koolstofverbindingen gaan uitscheiden. Deze verbindingen worden door de bacteriën weer als voedingstoffen opgenomen. Het interessante van het *A.tum.* systeem is dat het zijn T-DNA onder laboratorium omstandigheden ook over kan dragen naar andere planten, bacteriën, schimmels en gisten. Er is zelf één publicatie waarin men laat zien dat *A.tum.* zijn DNA kan overdragen naar humane cellen. Dit hebben wij echter niet kunnen reproduceren. Het lijkt er dus op dat het integratiemechanisme weinig gastheerspecifiek is. Dit maakt het mogelijk makkelijker om het aan humane cellen aan te passen dan het Mu integratiesysteem.

Bij het maken van T-DNA zijn minimaal twee *A.tum.* eiwitten betrokken: VirD1 en VirD2. Deze eiwitten herkennen samen één specifieke sequentie (border) die twee keer in A.tum voorkomt. Het T-DNA is tussen deze twee borders gelokaliseerd. VirD1 en VirD2 herkennen samen de border sequenties en zorgen ervoor dat het T-DNA wordt gemaakt waarna het integreert in het genoom van de gastheer. Om het integratiemechanisme van *A.tum.* geschikt te maken voor Adenovirus, hebben we een virale vector nodig dat het gen voor het VirD1 bevat, één virale vector dat het gen voor VirD2 bevat en een derde vector dat het T-DNA met borders bevat. Wanneer deze drie virale vectoren één humane cel infecteren, dan zal de cel VirD1- en VirD2-eiwit maken. VirD1 en VirD2 zorgen vervolgens voor de productie van T-DNA waarna het T-DNA integreert in het genoom van de humane cel. Door op dit T-DNA nu een gen voor een 'gezond' eiwit te plaatsen kunnen we ervoor zorgen dat na celdeling beide dochtercellen het gezonde eiwit blijven maken.

We hebben deze virale vectoren gemaakt en hebben laten zien dat de VirD1- en VirD2-eiwitten beide op de juiste plaats in de cel, de celkern, tot expressie komen. Op het T-DNA hebben we een gen geplaatst dat er voor zorgt dat de geïnfecteerde humane cel resistent wordt tegen een stofje dat normaal dodelijk is. Als het T-DNA integreert en we voegen dit stofje aan alle cellen toe, dan zullen alleen de cellen met de integratie overleven. Omdat de cellen delen zullen er kolonies ontstaan. De hoeveelheid kolonies die gevormd wordt is dan een maat voor de integratiefrequentie. Wanneer we humane cellen alleen infecteren met de T-DNA vector, dan kunnen we bepalen wat de spontane integratiefrequentie in deze cellen is. In onze proeven kwam dat overeen met al eerder gepubliceerde frequenties. Wanneer we humane cellen infecteerden met

zowel de T-DNA vector als de vector met VirD2, zagen we een 12-voudige verhoging van de integratie frequentie. VirD2 is het actieve enzym dat essentieel is voor het maken van T-DNA, maar kan dat normaal gesproken alleen doen samen met VirD1. Het was dus verrassend dat VirD2 zonder VirD1 al een verhoging van de integratiefrequentie gaf. Als mogelijke verklaring daarvoor dragen we aan dat Adenovirus in bepaalde tumorcellen al een beetje kan repliceren waardoor er enkelstrengs virale genomen kunnen ontstaan. Op enkelstrengs DNA kan VirD2 zonder hulp van VirD1 zijn werk doen. Wanneer we cellen infecteerden met de T-DNA vector en met de VirD1- en VirD2-vectoren, zagen we een 62-voudige toename van integratiefrequentie. Hieruit concluderen we dat VirD2 in staat is de integratiefrequentie van DNA te verhogen, in het bijzonder in aanwezigheid van VirD1. Verder hebben we ook laten zien dat het geïntegreerde DNA stabiel is en dat de expressie van genen op het geïntegreerde DNA niet uitgeschakeld wordt. We hebben alleen nog niet laten zien dat het geïntegreerde DNA ook daadwerkelijk het T-DNA is. Toen we hier naar keken bleek dat sequenties flankerend aan het T-DNA ook mee zijn geïntegreerd. Er is dus geen sprake is van integratie van alleen T-DNA maar van grotere stukken DNA. Het is belangrijk om te weten via welk mechanisme deze integraties hebben plaatsgevonden, maar daarvan hebben we nog geen resultaten.

Het integreren van DNA in het genoom van een patiënt heeft naast de eerder genoemde voordelen van verlenging van de expressieduur ook nadelen. Aangezien de plaats in het genoom waar het DNA integreert voornamelijk willekeurig is, is het bijvoorbeeld mogelijk dat de integratie een gen dat verantwoordelijk is voor de regulatie van celdeling verstoord. Dit kan een eerste stap zijn in de ontwikkeling van een tumor en wordt transformatie genoemd. In hoofdstuk **7** zetten we de voor- en de nadelen van integrerende vectoren naast elkaar en bekijken we het risico op transformatie aan de hand van data die in de loop van de jaren over integrerende virussen is verzameld.

Curiculum Vitae

Hendrik Jan Rademaker, born on February 4, 1976, Almelo. In 1993, he received his HAVO diploma at the St. Canisius in Almelo.

1993-1998:

Education at the Hogeschool Enschede
Hoger Laboratorium Onderwijs, specialization Medische Biochemie
Cum Laude

Training project of 9 months in the lab of Prof. dr. R.C. Hoeben, Department of Molecular Cell Biology, Sylvius Laboratory, Leiden University Medical Center, Leiden, The Netherlands. The aim of the project was to modify Human Adenoviruses so a specified part of viral DNA would integrate in the genome of human cells. Since HAds do not contain a dedicated integration mechanism, essential elements of the bacteriophage Mu integration system were tested for this purpose.

1998-2003:

PhD (Molecular Cell Biology): PhD in the lab of Prof. dr. R.C. Hoeben at the Leiden University Medical Center. The first project aimed at the generation of Human mobilization-resistant Adenoviruses (mrAds). The replication of these mrAds relies on factors that can not be complemented by wtAds. We characterized proteins of a Fowl Adenovirus that utilize replication initiation sites incompatible with Human Ads. The use of these alternative replication initiation sites in Human Ads creates mrAds. In combination with hybrid Human/Fowl replication proteins replication of these virusses is restored. This project was worked on in collaboration with Prof. dr. P.C. van der Vliet (University Medical Centre Utrecht and Centre for Biomedical Genetics).
The second project aimed at the incorporation of the *A.tum.* integration system in HAds. The goal was to obtain recombinant Ads capable of efficient integration of a specific part of the viral genome into human cells. This project was performed in collaboration with Prof. dr. P.J. Hooykaas (Leiden University).

2004-current:

Postdoc (Neuroscience): Currently working as a postdoc in the neuroscience department of the Erasmus Medical Center Rotterdam in the lab of Prof. dr. J.G.G. Borst. The project involves the generation of a kinesin based system to conditionally relocate synaptic vesicles to or from synaptic terminals and the *in vivo* imaging of synaptic release. For this purpose lentiviral vector technology and *in utero* electroporation technology was set up.