Recombinant adenoviral vectors (rAdVs) represent the most frequently used gene transfer system for preclinical and clinical approaches (Brunetti-Pierri et al., 2007; Morral et al., 1998). Different generations of rAdVs were developed by removing various viral coding regions and replacing them with foreign DNA. This greatly expanded the potential of rAdVs and to date rAdVs are extensively studied as oncolytics for the treatment of tumours and for treating genetic disorders.

To date 53 human wild-type adenovirus (wtAds) serotypes have been identified, which are divided into seven subgroups: A, B1, B2, C, D, E and F. They differ in their tropisms and their clinical manifestations. For instance subgroups B1, C and E cause respiratory infections, group B2 causes infections of the kidney and the urinary tract, group F viruses cause gastroenteritis and subgroup D viruses are associated with epidemic keratoconjunctivitis. The most commonly used adenovirus serotype for generation of rAdVs is represented by the human adenovirus serotype 5 (Ad5) from subgroup C. However, during recent years there has been an increased interest in the generation of rAdVs other than those based on Ad5 (Bromberg et al., 1998; Yang et al., 1995). This is because Ad5 is restricted by its tropism and there is a high prevalence of neutralizing antibodies against Ad5 within the human population. Therefore, by exploring the complete spectrum of all available human wtAds one could benefit from the diverse tissue tropism available and one may circumvent pre-existing immunity.

All rAdVs used for genetic engineering of cells share the serotype-specific inverted terminal repeats (ITRs) flanking both sides of the adenovirus genome as one common feature in their genomic composition. It has been established that the ITRs play an important role in adenovirus DNA replication. Dependent on the serotype, the ITRs are approximately 160 bp in length and sequence comparisons revealed that the first 50 bp from the ends in particular contain sequences that have been well conserved during adenovirus evolution (Ishino et al., 1987; Tolun et al., 1979). There is evidence that transcriptional activity is located within the ITRs of Ad5 (bp 1–103) (Yamamoto et al., 2003), nucleotide sequences 1–190 and 1–342 (Yamamoto et al., 2003), and downstream of the adenoviral ITR sequences within the packaging/enhancer region (bp 194–458) (Hearing et al., 1987; Rubinchik et al., 2001; Shi et al., 1997). This feature is important because transcriptional activity derived from the ends of the adenovirus genome can influence the performance of a given transgene inserted into a rAdV genome. Towards this end it was shown that even highly tissue-specific promoters delivered by rAdV display altered transcriptional activity (Babiss et al., 1986; Hatfield & Hearing, 1991; Imler et al., 1996; Shi et al., 1997). Additionally, in the relatively rare case of integration of the vector genome into the chromosomal host DNA (Harui et al., 1999; Stephen et al., 2008), a transcriptionally active ITR may force or downregulate expression of nearby genes or even oncogenes. Although the left arm of Ad5, including the ITR and
packaging/enhancer region, has been studied in the past, there is virtually no information about ITRs with respect to transcriptional activity from human adenovirus serotypes other than Ad5. There are only studies based on Ad5 (Yamamoto et al., 2003) and porcine adenovirus type 3 (Xing & Tikoo, 2005, 2006) indicating that promoter activity resides in the ITR itself.

Herein we investigated for the first time in a plasmid-based system whether ITRs from human adenovirus subgroups A, B1, B2, C, D, E and F show transcriptional activity in various cell lines. To cover the complete spectrum of all subgroups we have chosen one serotype from each group [Ad12(A), Ad7(B1), Ad11(B2), Ad5(C), Ad17(D), Ad4(E) and Ad41(F)] and generated constructs in which luciferase expression is dependent on the transcriptional strength of the respective ITR. For amplification and cloning of the different ITRs, primers were designed to clone either the forward or the reverse orientation of the ITR. Respective primers and the accession number of the different adenovirus serotypes are provided in Supplementary Table S1 (available in JGV Online). All vector constructs tested in the present study were based on the pGL3-Control vector (Promega), in which luciferase expression is under the control of the Simian virus 40 (SV40) promoter (Fig. 1a). This vector served as a positive control for reporter gene expression. The negative control vector pGL3 Δ SV40 (Fig. 1a) was generated by deleting the SV40 promoter. In order to analyse all possible directions in which transcription can run, either into the transgene expression cassette or, in the case of integration, into the chromosomal DNA, the ITRs were cloned upstream of the reporter gene in forward and reverse orientations (Fig. 1a and Supplementary Methods, available in JGV Online). To search for potential transcription-factor binding sites in the ITRs which were

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**Fig. 1.** Vector constructs and TATA-box predictions within the inverted terminal repeats (ITRs). (a) Final vector constructs for analysis of the transcriptional activity of adenoviral ITRs and the respective adenovirus subgroups. pSV40, Promoter of the Simian virus 40; polyA, SV40 polyadenylation signal; pPGK, phosphoglycerate-kinase promoter; SV40E, Simian virus 40 enhancer. The respective restriction enzyme nucleases used for generation of the constructs are indicated. For cloning procedures please refer to the Supplementary Methods. (b) Search for transcription-factor binding sites in the ITRs utilizing a transcription element search system. Multiple transcription factor binding sites (TFBS) were identified and are shown schematically as TFBS X, Y and Z. For all ITRs derived from different serotypes nucleotides 10–16 (TAATAT) could serve as a TATA box.
used in our study, we used the ‘Transcription Element Search System’ (TESS), a free online tool provided by the University of Pennsylvania (http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME). In all ITRs derived from different serotypes, nucleotides 10–16 (TAATAT) could serve as a TATA box (Fig. 1b) and multiple transcription factor binding sites (e.g. for Oct-1, NFI, TBP, SP1 and ATF) were predicted.

In order to analyse whether the transcriptional potential of the ITRs is cell-type dependent, different cell lines were used. First, we tested cells which are permissive to adenovirus infection such as HEK293 cells derived from human embryonic kidney, HeLa cells derived from a cervical carcinoma and A549 cells originating from lung epithelial cells. Because of the natural liver tropism of Ad5, we also studied transcriptional activity in the hepatoma-derived human liver cell line Huh7. Ad41 displays a distinct tropism for the intestinal tract and therefore we analysed transcription in the colon carcinoma cell line HCT116. Furthermore, the adult retinal pigment epithelia cell line ARPE-19 was studied because of the natural tropism of some adenoviral serotypes for the eye (Ad17 and Ad4). All cell lines were cultivated as previously described (Ehrhardt et al., 2005; Maucksch et al., 2008) or according to the instructions of the American tissue culture collection.

The potential transcriptional activity of the respective ITRs based on the constructs displayed in Fig. 1(a) was determined by measuring firefly-luciferase activity utilizing a dual luciferase reporter assay. (For details please refer to the Supplementary Methods.) In the adenovirus-permissive cell lines HEK293, HeLa and A549, we detected robust promoter activity from the ITRs from subgroup A, C and F if they appear in a forward orientation (Fig. 2a–c). Notably, expression levels were comparable or even higher compared with the strong SV40 promoter within the vector pGL3-Control. Most other forward-orientated ITRs promote minor changes (ITR 17) or background luciferase-expression levels (ITRs 7 and 4). In contrast, in the reverse orientation only serotypes 11 and 17 are transcriptionally active in HEK293, HeLa and A549 cells, whereas all other ITRs seem to inhibit luciferase expression (ITR 4, 5, 7, 12 and 41), particularly ITR 12 which has no detectable luciferase expression (Fig. 2a–c). Similar results were obtained in the liver-specific Huh7 cells and the colon carcinoma cell line HCT116 (Fig. 2d, e). In the forward orientation ITRs from adenovirus serotypes 12, 5 and 41 show strong luciferase expression. In the reverse orientation ITRs 11 and 17 display slight promoter activity and the other ITRs negatively interfere with transcription. We also investigated transcriptional activity, in the retinal pigment epithelial cell line ARPE-19. In this case, the ITRs from subgroups A, C and F show the highest activity in comparison with the negative control lacking the SV40 promoter (Fig. 2f). In concordance with the results obtained in all other cell lines the reverse-orientated ITRs from adenovirus serotypes 11 and 17 display robust activity and ITRs 5, 7, 12, 41 display an inhibitory function (Fig. 2f). However, in contrast to results obtained in all other cell lines...
lines, we measured slightly enhanced luciferase expression levels in ARPE-19 cells for the ITR from subgroup E (ITR 4) when it was cloned in the reverse orientation (Fig. 2f). The reason for this finding remains to be elucidated.

In conclusion, we provide evidence that the transcriptional activity of the ITRs is not tissue dependent, as comparable luciferase expression patterns were observed in all cell lines analysed. To our surprise we observed strong transcriptional activity for ITRs 5, 12 and 41 in all cell lines. These ITRs were all active in the forward orientation but not when inserted in the reverse orientation. In this latter case no promoter activity could be measured, indicating that these ITRs somehow act as inhibitors for transgene expression; furthermore, background luciferase expression diminishes (Fig. 2). In contrast, ITR 11 drives sufficient luciferase expression only when in the reverse direction but not when in the forward orientation. However, future studies should analyse transcriptional activity in the context of the virus.

Our results may also have important implications for the design of rAdVs based on a subgroup other than subgroup C. For instance, there is evidence that the position of the transgene expression cassette inserted within the rAdV genome has an impact on the expression level of the transgene. It was shown that an enhancer near the 5’ end could lead to leaky expression from a tissue-specific promoter even if it was in non-target cells (Babiss et al., 1986; Shi et al., 1997). Furthermore, it has been reported that sequences around the ITR can reduce promoter activity by up to a factor of 50 (Shi et al., 1997). Therefore, ITRs showing low or no inward transcriptional activity into the adenoviral genome may be advantageous for optimized vector design. To avoid interference between adjacent genes without a termination signal (Proudfoot, 1986) when inserting a transgene expression cassette into the rAdV genome, ITR sequences derived from an adenovirus serotype which has no or less effect on transcription initiation could be beneficial, especially when using tissue-specific or controllable promoters.

To begin to find transcription start sites within our constructs we performed initial experiments based on 5’ RACE (for detailed information see Supplementary Methods). For Ad5 and Ad7 ITR we found transcription start sites (TSS) at nucleotide positions 258 and 254 of the pGL3-Control vector, located 26 and 30 nt upstream of the luciferase start codon and 41 and 37 nt downstream of the ITR, respectively. This indicates that TSS are located at this position and therefore there is promoter activity in the ITR. However, it is of note that we used a plasmid-based approach and that the situation could be different in the context of an adenovirus due to viral proteins being bound to the ITRs during infection.

To address the question of whether ITRs from various adenovirus serotypes can influence a nearby promoter, we analysed the influence of the ITRs on the transcriptional activity of the ubiquitously expressed PGK promoter (pPGK). Towards this end, the PGK promoter was cloned...
downstream of the forward-orientated ITRs from each adenovirus subgroup (Fig. 1a). The experimental set-up was identical to the experiments shown in Fig. 2. Unexpectedly, we detected that almost every ITR upstream of the pPGK significantly reduced luciferase expression to background levels or even less (Fig. 3). With the exception of results obtained in A549 cells, only the ITR 7 from subgroup B1 seems to have no or only minor influence on the activity of pPGK in the cell lines HEK293, HeLa, Huh7 and HCT116. This is in agreement with results obtained for ITR 7 without the pPGK. In all cell lines tested, serotype 7 shows either no or only a minor influence on the transcriptional level in forward as well as in reverse orientations (Fig. 2). This observation remains to be elucidated, but nevertheless this ITR seems to be a good candidate for the optimized design of rAdVs. However, it also remains to be elucidated whether this phenomenon is a special feature of the PGK promoter and whether another promoter may be differently influenced by the ITRs in terms of its transcriptional activity.

In summary, we show that the ITRs from several adenovirus serotypes on their own enhance or inhibit transcriptional activity in a tissue-independent manner, and that this activity can downregulate nearby promoters. With respect to gene therapeutic approaches our results indicate that transcriptional activity from the forward-orientated ITR into the adenoviral genome could influence the transgene expression cassette within the rAdV genome. One may also speculate that convergent promoter activity from the internal promoter and the ITR may occur, which then leads to formation of dsRNAs that may induce gene silencing due to RNA interference, thus influencing the virus life cycle. Moreover, the outward-orientated transcriptional activity derived from ITRs may have a special importance for adenoviruses that show integration activity into the host genome of infected cells, such as Ad12 into hamster cells (Sawada et al., 1979; Sutter et al., 1978). In our experiments ITR 12 from subgroup A results in strongly inhibited luciferase expression when cloned in the reverse orientation, suggesting that this activity may result in inhibition of nearby genes. However, future studies should address whether the influence of the ITRs on transcriptional activity can be translated from plasmid-based studies into the context of an adenovirus.

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