

Conditional Repression of the E2 Transcription Unit in E1-E3-Deleted Adenovirus Vectors Is Correlated with a Strong Reduction in Viral DNA Replication and Late Gene Expression In Vitro

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An E1-E3-deleted recombinant adenovirus vector expressing the hybrid protein TetR-KRAB has been produced. In this virus, AdTG9562, the E2 transcription is regulated by TetR-KRAB and *tetO* sequences inserted in *cis*. In the absence of tetracycline, a strong reduction in E2A gene expression, viral DNA replication, and late gene expression was observed in noncomplementing A549 cells, and a reduction in viral growth was seen in the E1-expressing 293 cells. In contrast, there was no repression in the presence of the regulator tetracycline. We propose that regulation by TetR-KRAB is a valuable tool with which to study the effects of viral gene expression in vitro.

Recombinant adenoviruses are interesting systems for human gene therapy applications because they are efficient vectors for the in vivo and in vitro transfer of heterologous genes into dividing and nondividing cells (32). However, in vivo transduction of first-generation adenovirus vectors with E1 and E3 deleted leads only to transient expression of transgenes accompanied by substantial inflammatory and immune responses (8, 29). The destructive immune response was ascribed to the cellular immunity against early and late viral antigens, which are expressed even in the absence of the transactivation functions of E1A (34). Therefore, adenovirus vectors were further crippled in early region 4 (E4) or 2A (E2A) to reduce any residual expression of viral antigens in target cells. The resulting second-generation adenovirus vectors have deletions of either E4 or E2A and have to be propagated in the appropriate complementation cell lines providing E1 and E4 (20, 33, 36) or E1 and E2A (12) functions in *trans*. Alternatively, adenovirus vectors have been described that express a thermosensitive E2A gene product. These second-generation adenovirus vectors based on Ad5Hts.125 (10) showed a reduction in the cytotoxic T-lymphocyte response at the semipermissive temperature of 37°C and an improved persistence of transgene expression in mouse liver and lung (9, 35). However, the latter observation could not be confirmed, since the *ts125* mutation was not sufficient to prolong the expression of canine factor IX in dogs with hemophilia B after application of the respective adenovirus vector (11).

The E2 region encodes proteins vital to viral DNA replication (for review, see references 2, 16, and 19). The E2B region provides the precursor terminal protein (80 kDa) and the viral DNA polymerase (140 kDa); the gene product of E2A is the abundant nuclear DNA-binding protein (DBP [72 kDa]) (1, 19). Studies of DBP functions have been supported by temperature-sensitive mutants, such as Ad5Hts.125, that are unable to replicate viral DNA at a nonpermissive temperature (39°C) (10). Besides its role during viral DNA replication, there have been regulatory functions ascribed to DBP, such as repression of transcription from the E4 region (25) and transcriptional activation of the major late promoter (3).

E2 gene expression is driven from two promoters active early and late during infection. The basal activity of the E2 early promoter (position 27092) depends on nonconsensus TATA motifs and ATF-2 and E2F binding sites (31). E1A-mediated transcriptional activation is due to (i) ATF-2 binding to the E2 early and E4 promoters (22) and (ii) dissociation of E2F-DP1-Rb complexes and association of E2F-DP1 with a 19-kDa gene product of E4 open reading frame 6/7 which enhances the stable cooperative binding of E2F to its binding sites on the E2 early promoter (17, 24). At intermediate times after infection, E2A transcription switches to the E2 late promoter (position 25354). This promoter consists of a TATA-like sequence, Spl binding sites, and a CAAT box and is not sensitive to E1A regulation (31).

In this study, we describe a recombinant adenovirus vector that modulates E2A gene expression via repression of basal and, in the presence of E1A, activated E2 promoter function. We used a system described by Deuschle et al. (6) based on the strong transcriptional repression functions of the Krüppel-associated box (KRAB) domain of the human Kox zinc finger protein (23). The evolutionarily conserved KRAB domain contains a 75-amino-acid region of homology present in the N termini of a large number of Krüppel-type zinc finger proteins. In vitro transcription assays have indicated that the KRAB domain is able to repress both activated and basal transcriptional activity and that repression requires a TATA box located within the promoter (26). Fusion of the KRAB domain to the *Tn10*-derived procaryotic tetracycline repressor protein TetR allows targeting of the KRAB-mediated repression functions: TetR-KRAB can bind to the tetracycline operator (*tetO*) sequences, and binding is regulated by tetracycline (13). It was shown that TetR-KRAB can repress different eucaryotic promoters by binding even far upstream (up to 3.6 kb) of the transcriptional start site (6). We took advantage of this feature, since it is not possible to manipulate the E2 promoters close to the cap sites because of the overlap with the major late open reading frame L4 (Fig. 1).

We produced the recombinant adenovirus vector AdTG9562 that carries a cytomegalovirus (CMV) promoter-driven TetR-KRAB expression cassette in place of the E1 region and the *tetO* heptamer introduced in place of the E3 region 1,500 and 2,638 bp upstream of the cap sites of E2 early and late promoter, respectively, without disturbing the L4 or L5 region

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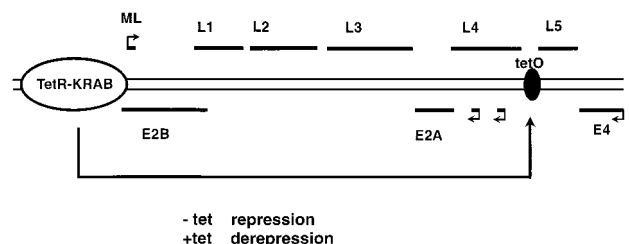


FIG. 1. Schematic representation of AdTG9562 and its regulation of E2 gene expression: the recombinant adenovirus vector derived from adenovirus type 5 (5) carries a CMV promoter-driven expression cassette for the transcriptional silencer TetR-KRAB (6) in place of the E1 region. The TetR domain of this fusion protein allows binding to a heptamer of tetracycline (tet) operator sequences (*tetO* [13]) inserted *in cis* in place of the E3 region at position 28592. Upon binding, the KRAB domain confers repression of E2 early (cap site position 27092) and E2 late (cap site position 25954) promoters. The binding of TetR-KRAB, and subsequently the effect, can be reverted in the presence of the regulator tetracycline.

(Fig. 1). E2A gene expression, viral DNA replication, and late gene expression were studied in the absence and presence of the regulator tetracycline in noncomplementing A549 cells (30) and E1-complementing 293 cells (15). We observed a conditional reduction of viral gene expression in A549 cells as well as a modulation of viral growth in 293 cells.

Construction and propagation of AdTG9562. The viral DNA of AdTG9562 (Fig. 1) was generated by homologous recombination in *Escherichia coli* cells (4) starting with the adenovirus type 5 genome (Ad5) introduced into pPolyII (21) (pTG3602). Digestion of pTG3602 with *Bam*HI and religation led to a construct carrying adenovirus sequences from the unique *Bam*HI site at position 21562 (nucleotide numbers refer to positions on the Ad5 genome according to reference 5) to the 3' end at position 35935; the E3 region was deleted by eliminating an *Xba*I fragment (positions 28592 to 30470 [pTG9524]). The *tetO* heptamer derived as an *Xho*I (T4Pol)-*Sma*I fragment from pUHD10-3 (13) was inserted into the remaining *Xba*I (Klenow) site at position 28592 (pTG9549). A *Bam*HI fragment carrying Ad5 sequences from position 1 to the unique *Bam*HI site at position 21562 with E1 replaced by a *lacZ* expression cassette was inserted into the unique *Bam*HI site of pTG9549 reconstituting an adenovirus vector genome (Δ E1 *lacZ* Δ E3 *tetO* [pTG9550]). The *lacZ* expression cassette was replaced by a CMV promoter-driven expression cassette for TetR-KRAB by homologous recombination between pTG9550 cut by *Cla*I and a *Pac*I-*Sma*I fragment from pTG9559 according to the technique described by Chartier et al. (4), leading to the final construct, pTG9562. The replacement vector pTG9559 carries Ad5 sequences from positions 1 to 5788 with a deletion of E1 from positions 459 to 3328 replaced by an *Xho*I-*Hind*III fragment of pCMVtetR-KRAB (6). The recombinant viral DNA was released from pTG9562 by digestion with *Pac*I and transfected into E1-complementing 293 cells in the presence of tetracycline (1 μ g/ml). Plaques appeared 6 days after transfection, and virus was purified according to the method of Graham and Prevec (14). The titer of infectious virus was determined by counting of 293 cells expressing DBP 16 h after infection monitored by immunofluorescence analysis. AdTG9562 preparations reached titers of 10^{11} infectious units (iu)/ml.

Conditional reduction of E2A and late gene expression. The potential for regulation of E2A basal gene expression with AdTG9562 was evaluated in the noncomplementing human lung carcinoma cell line A549.

A549 cells were infected with AdTG9562 or AdTG9545—an

adenoviral vector with E1 deleted (positions 459 to 3328)—at multiplicities of infection (MOIs) of 180 and 60 iu/cell. Cells were harvested 3 days after infection and total protein extract was monitored for DBP expression by Western blotting. Figure 2A shows that DBP production was reduced by 90% in cells infected with AdTG9562 in the absence of tetracycline; the DBP expression level in the presence of tetracycline was comparable to that observed after infection with AdTG9545 in the

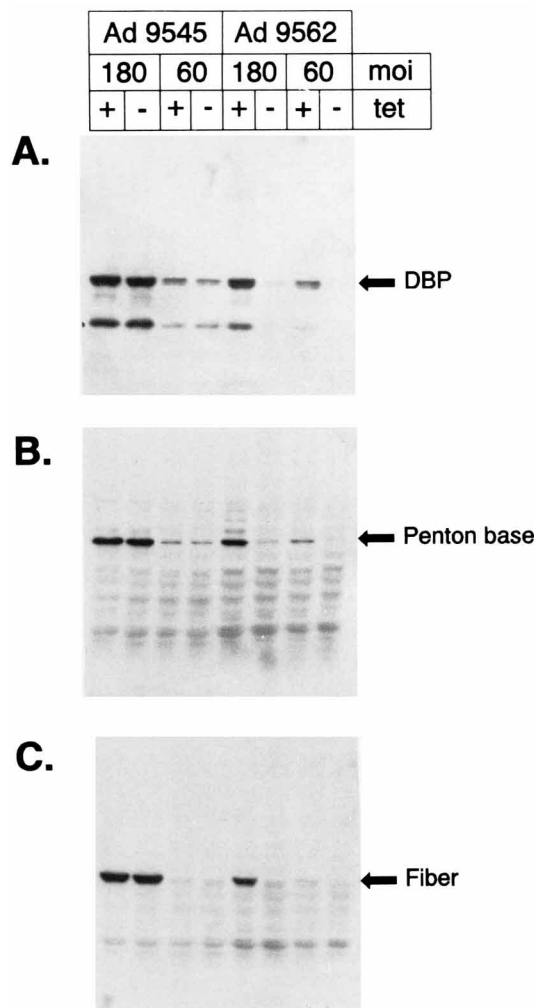


FIG. 2. Reduction of DBP, fiber, and penton base synthesis in A549 cells infected with AdTG9562 in the absence of tetracycline (tet). A549 cells were infected with AdTG9562 and AdTG9545 at MOIs of 180 and 60 iu in absence (–) and presence (+) of tetracycline (tet [1 μ g/ml]). The titer of the virus preparations had been determined as infectious units per milliliter: 293 cells were seeded on eight chamber glass slides (LabTek, 2×10^5 cells/chamber). Confluent monolayers were infected with defined volumes of virus preparations. The cells were fixed 16 h after infection before the onset of a second round of infection and stained with a monoclonal antibody specific for DBP (B6 α 72K [27]) and a fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G. Extrapolation of the number of cells expressing DBP with respect to the dimension of the chamber and magnification by the immunofluorescence microscope gave the number of particles in the virus preparation that were able to infect 293 cells. A549 cells were harvested 3 days after infection, and total protein was extracted. Western blot analysis was performed with primary antibodies directed against DBP (monoclonal antibody B6 α 72K [27]; the lower band is a degradation product and corresponds to the C-terminal domain of DBP) (A), penton base (monoclonal antibody, kind gift from P. Boulanger) (B), and fiber (polyclonal antibody E642 [18]) (C). Secondary antibodies directed against mouse or rabbit immunoglobulin linked to horseradish peroxidase allowed specific immunostaining with the enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham).

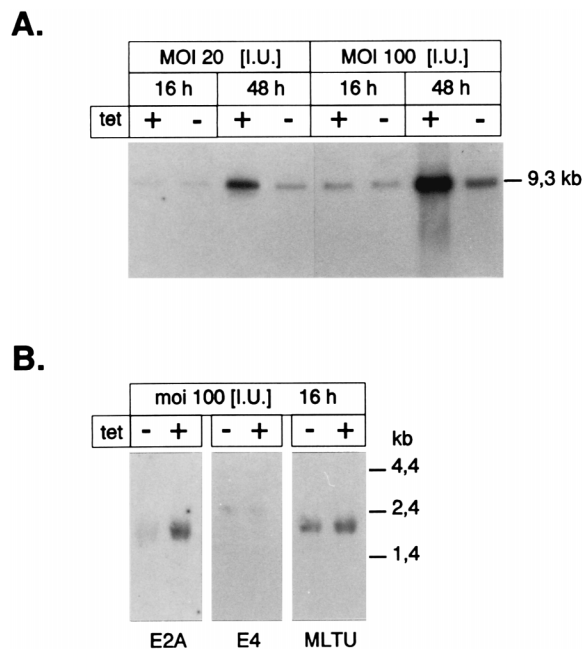


FIG. 3. (A) TetR-KRAB reduces replication of viral DNA in A549 cells. A549 cells were infected with AdTG9562 at MOIs of 20 and 100 iu/cell. Cells were harvested 16 and 48 h after infection. Southern blot analysis of Hirt DNA cut by *Xho*I was performed with an oligonucleotide probe, OTG 6931 (positions 32920 to 32940). (B) TetR-KRAB reduces specifically the steady-state level of E2A mRNA. Total RNA was extracted from A549 cells 16 h after infection with AdTG9562 at an MOI of 100 iu/cell in the absence (-) and presence (+) of tetracycline (tet [RNA NOW; Biogentex, Inc.]). Northern blot analysis of viral RNA was performed with oligonucleotide probes specific for E2A, E4 and the major late transcription unit (MLTU; fiber) mRNAs. The E2A mRNA was 1.8 kb, the E4 mRNA was 2.3 kb, and the fiber mRNA was 1.9 kb.

absence and presence of tetracycline. The same pattern of expression was observed for two late viral proteins—penton base and fiber (Fig. 2B and C). We conclude that tetracycline had no effect on DBP or late gene expression in A549 cells infected with AdTG9545. However, tetracycline did regulate both DBP and late gene expression in A549 cells infected with AdTG9562, which carries the TetR-KRAB gene and *tetO* sequences. Moreover, AdTG9562 was shown to be phenotypically stable, since similar results were obtained with virus that had been passaged 10 times on 293 cells in the absence of tetracycline (data not shown).

Conditional repression of E2A transcription and reduction of viral DNA replication. To study the mechanism of the repression of viral protein synthesis in more detail, A549 cells were infected with AdTG9562 at MOIs of 20 and 100 iu/cell. Cells were harvested 16 and 48 h after infection, and viral DNA was extracted and submitted to Southern blot analysis (Fig. 3A). Significant viral DNA synthesis had taken place 48 h after infection in the presence of tetracycline. In contrast, DNA replication was approximately fivefold less efficient in tetracycline's absence.

The amounts of viral DNA 16 h after infection were comparable in the absence and presence of tetracycline (Fig. 3A). Under these conditions, in which viral DNA replication had not significantly changed the amounts of templates for gene expression, viral RNA was analyzed: A549 cells were infected with AdTG9562 at an MOI of 100 iu/cell, and total RNA was prepared 16 h after infection. A reduction in E2A mRNA of 90% was observed in the absence of tetracycline (Fig. 3B). Transcription from the E4 region was analyzed with oligonucleotide probes directed against sequences upstream of the E4

poly(A) signal (positions 32940 to 32859) which are present on all E4 transcripts (7). A 2.3-kb RNA could be detected with expression levels unaffected by tetracycline (Fig. 3B). This indicates that tetracycline-regulated effects of TetR-KRAB did not influence transcription from the E4 region. However, transcription of fiber mRNA driven from the major late promoter was slightly reduced in the absence of tetracycline (Fig. 3B). This reduction could be ascribed to the regulatory functions of DBP (4), which are disturbed if E2A gene expression is reduced. Although we have no proof, the distance of approximately 21 kb between the *tetO* sequences and the major late promoter argues against a direct regulatory effect of *tetO*-bound TetR-KRAB on this promoter. So far, we have no indication that free TetR-KRAB influences expression driven from the major late promoter: transfection of A549 cells with the expression plasmid pCMV tetR-KRAB (6) followed by a superinfection with AdTG9545 at an MOI of 200 iu/cell led to levels of DBP and late gene expression that were comparable in transfected and mock-transfected cells (data not shown). Furthermore, fiber expression was comparable in A549 cells infected with E1-deleted adenovirus vector or AdTG9562 in the presence of tetracycline (Fig. 2). The results shown in Fig. 3B confirm that in the absence of tetracycline, TetR-KRAB can specifically repress basal transcription from the E2 promoter in the absence of E1A (Fig. 3B). Reduced levels of DBP lead to a reduction of viral DNA synthesis 48 h after infection (Fig. 3A), which in turn results in reduction of further DBP and late gene expression 3 days after infection (Fig. 2).

Comparison of AdTG9562 with double-deleted adenovirus vectors in noncomplementing A549 cells. Viral gene expression in AdTG9545-infected A549 cells was compared with that of double-deleted adenovirus vectors: AdTG9546, with deletions of E1 and E4, and AdTG9592 with deletions of E1 and E2A. The E4 deletion in AdTG9546 comprises positions 32994 to 34998; in AdTG9592, the whole DBP coding region from positions 22441 to 24035 was deleted (unpublished results). The double-deleted adenovirus vectors are replication-deficient in 293 cells (see Fig. 5B) and were propagated in the respective complementation cell lines (unpublished results).

Comparison of levels of DBP expression in A549 cells 3 days postinfection with the different adenovirus vectors at the MOI of 200 iu/cell confirmed the conditional repression of DBP expression regulated by tetracycline (Fig. 4, lanes 1 and 2 [AdTG9562]). The amounts of DBP produced by AdTG9562 in the presence of tetracycline and the E1-deleted control vector were comparable (Fig. 4, lanes 1 and 5). DBP expression of AdTG9546 ($\Delta E1\Delta E4$) was reduced compared to that seen with AdTG9545 ($\Delta E1$) (Fig. 4, lanes 3 and 5), while DBP expression was absent, as expected, in cells infected with AdTG9592 ($\Delta E1\Delta E2A$) (Fig. 4, lane 4).

We conclude that viral gene expression of AdTG9562 in the presence of tetracycline is comparable to that seen with AdTG9545 ($\Delta E1$) in noncomplementing A549 cells. In the absence of tetracycline, AdTG9562 resembles the E1-E4-deleted adenovirus vectors with respect to the significant reduction of DBP expression 3 days after infection. However, if reduction of E2A gene expression due to repression by TetR-KRAB or as a result of the deletion of E4 is not complete, DBP can still be detected.

Comparison of AdTG9562 with double-deleted adenovirus vectors in E1-complementing 293 cells. We asked whether TetR-KRAB can repress the E2 promoter in the presence of E1A transactivating functions and thereby modulate the viral amplification in 293 cells. 293 cells were infected with AdTG9562 at an MOI of 0.01 iu; the cells were harvested 5

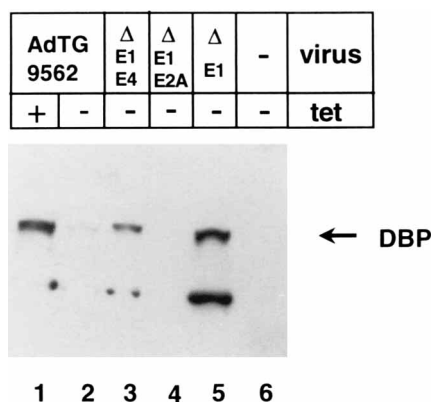


FIG. 4. Comparison of levels of viral gene expression in A549 cells infected with AdTG9562, AdTG9545 (Δ E1), AdTG9546 (Δ E1-E4), and AdTG9592 (Δ E1-E2A) at an MOI of 200 iu/cell. Cells were harvested 3 days after infection, and total protein was extracted. Western blot analysis was performed with the monoclonal antibody B6 α 72K directed against DBP (27); the lower band is a degradation product and corresponds to the C-terminal domain of DBP. tet, tetracycline.

days after infection. Viral DNA replication was reduced in the absence of tetracycline by 80% (Fig. 5A). These results show that viral amplification is modulated, indicating that TetR-KRAB is able to repress the E2 promoter activity even in the presence of the transactivating E1A functions. The extent of the modulation was dependent on the MOI and duration of infection; viral DNA harvested 2 days after infection of 293 cells at the MOI of 1 per cell revealed only a moderate twofold reduction of viral replication as measured by Southern blot analysis (Fig. 5B, lanes 7 and 8). In contrast, viral replication could not be detected in 293 cells infected with E1-E4- or E1-E2A-deleted adenovirus vectors (Fig. 5B, lanes 5 and 6).

In summary, an E1-E3-deleted adenovirus vector, AdTG9562, has been generated that allows conditional repression of basal E2A gene expression. The E2 promoter is modulated even in the presence of E1A, since virus growth is reduced in 293 cells. However, compared with E1-E4-deleted and E1-E2A-deleted adenovirus vectors, the effect of conditional repression by TetR-KRAB seems to be more pronounced in A549 cells than

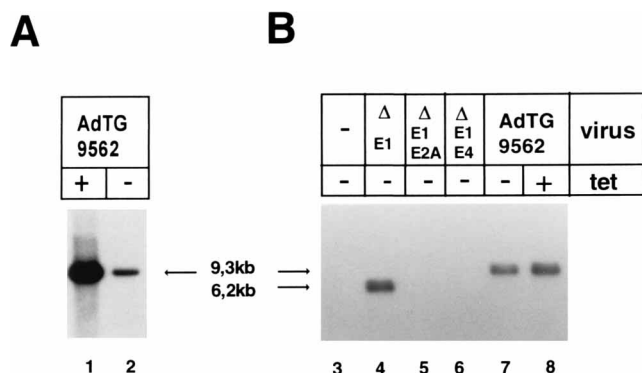


FIG. 5. (A) Modulation of virus propagation in the presence of E1. 293 cells were infected with AdTG9562 in the absence (-) and presence (+) of tetracycline (tet) at an MOI of 0.01 iu/cell; Hirt DNA was harvested 5 days after infection. (B) Leakiness of viral replication. 293 cells were infected with AdTG9562, AdTG9545 (Δ E1), AdTG9546 (Δ E1-E4), and AdTG9592 (Δ E1-E2A) at an MOI of 1 iu/cell. The construction and propagation of AdTG9546 and AdTG9592 will be described elsewhere. Hirt DNA was harvested 2 days after infection. Southern blot analysis of viral DNA digested by *Xho*I was performed with an oligonucleotide probe OTG6931 (positions 32920 to 32940).

in the E1-complementing 293 cells. This difference might be due to the presence of E1A, which is involved in the transcriptional activation of the E2 early promoter in 293 cells (17, 22, 24). One approach to reduce the leakiness of TetR-KRAB-mediated repression of the E2 promoter could be the deletion of the E4 open reading frame 6/7 region in the context of AdTG9562, since this would interfere with the binding of E2F to the E2 early promoter (17, 24).

AdTG9562 is a novel tool with which to study the effects of adenovirus gene expression in target cells *in vitro*; in the human lung carcinoma cell line A549, the E2A and E2B region is expressed, allowing for replication of viral DNA after infection with an MOI as low as 20 iu/cell. The increase in the amounts of the viral DNA templates augments the amounts of early and late viral proteins expressed in the target cell. Repression of transcription from the E2 promoter reduces the amount of DBP (Fig. 2A and 3B) and probably also reduces the amounts of DNA polymerase and precursor terminal protein. As a consequence, viral DNA replication and late gene expression are decreased. Reduced activity of the major late promoter, probably due to the repression of DBP expression, might contribute to this effect.

We do not propose to use AdTG9562-derived vectors for gene transfer application *in vivo*, since (i) repression of DBP expression is not complete, (ii) the prokaryotic TetR domain of TetR-KRAB might be immunogenic, and (iii) less-leaky double-deleted adenovirus vectors exist. However, the TetR-KRAB-regulated adenovirus vector could contribute to evaluation of the effect of reduced residual gene expression *in vivo*: AdTG9562 can be compared with a first-generation adenovirus vector with E1 deleted with respect to viral gene expression in the presence of tetracycline (Fig. 2); the pattern of reduced E2A and late gene expression in the absence of tetracycline was comparable to that obtained with a second-generation adenovirus vector with deletions of E1 and E4 (Fig. 4).

The comparison of adenovirus vectors with deletions of E1 and E4 or E2A is hampered by differences in the ratios of physical particles, infectious units, and PFU (22a). These ratios vary between first- and second-generation adenovirus vectors, since insufficient transcomplementation of viral functions increases the number of viral particles that either cannot infect or cannot allow for viral amplification after infection. However, comparison of first- and second-generation adenovirus vectors in animal models will have to be based on one parameter (e.g., infectious units) introducing major differences with respect to other parameters (e.g., physical particles). With AdTG9562, this problem is circumvented, since the same adenovirus preparation can be applied to animals that are treated with or without tetracycline (28) to provide conditions under which only viral DNA replication and gene expression are modulated.

In conclusion, we propose that TetR-KRAB-mediated regulation of gene expression can become an important tool for basic virology studies. The possibility of specifically modulating the expression level of viral proteins allows us to analyze their function in the context of a viral infection *in vitro*. With AdTG9562, we were able to dissect the effects of residual viral gene expression *in vitro*. Moreover, the study of the effects of gene expression from other complex viral vectors such as recombinant CMV or herpes simplex virus will be greatly facilitated.

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