Inducible Overexpression of a Toxic Protein by an Adenovirus Vector with a Tetracycline-Regulatable Expression Cassette†

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We have constructed two new adenovirus expression cassettes that expand both the range of genes which can be expressed with adenovirus vectors (AdV) and the range of cells in which high-level expression can be attained. By inclusion of a tetracycline-regulated promoter in the transfer vector pAdTR5, it is now possible to generate recombinant adenoviruses expressing proteins that are either cytotoxic or that interfere with adenovirus replication. We have used this strategy to generate a recombinant adenovirus encoding a deletion in the R1 subunit (R1Δ(Δ2-357)) of the herpes simplex virus type 2 ribonucleotide reductase. Cell lines expressing the tetracycline-regulated transactivator (tTA) from an integrated vector or following infection with an AdV expressing tTA are able to produce ΔR1 protein at a level approaching 10% total cell protein (TCP) when infected with AdSTRA1R1 before they subsequently die. To our knowledge, this is the first report of the overexpression of a toxic gene product with AdV. We have also constructed a new constitutive adenovirus expression cassette based on an optimized cytomegalovirus immediate-early promoter-enhancer that allows the expression of recombinant proteins at a level greater than 20% TCP in nonpermissive cell lines. Together, these new expression cassettes significantly improve the utility of the adenovirus system for high-level expression of recombinant proteins in animal cells and will undoubtedly find useful applications in gene therapy.

The adenovirus expression system (AES) is routinely used both for protein production and for gene transfer experiments (reviewed in references 2, 4, 5, 14, 31, and 34). Among the attractive features of the AES are the high gene transfer capacity of adenovirus vectors (AdV) in a wide range of cell types both in vitro and in vivo and very efficient transgene expression. We recently reported on an adenovirus major late promoter (MLP) expression cassette, pAdBM5, which allows for the production of heterologous proteins in the human 293 cell line at levels of 15 to 20% total cell protein (TCP) (23). Successful overexpression with pAdBM5 and with similar vectors during the course of adenovirus replication in permissive 293 cells is due both to the efficiency of the expression cassette and to gene amplification in combination with the selective expression of viral genes during the late phase of the adenovirus lytic cycle. Thus, the level of transgene expression with AdV having deletions of E1 following infection of nonpermissive cells even at a high multiplicity of infection (MOI) is much lower than that in 293 cells. For gene transfer experiments in nonpermissive cells, most AdV make use of the cytomegalovirus (CMV) immediate-early (IE) promoter-enhancer in the expression cassette, since this promoter is one of the strongest in a wide range of cell types (reviewed in references 22 and 36). Despite this fact, AdV with CMV-based expression cassettes rarely produce protein exceeding 1 to 2% TCP after infection of either nonpermissive cells or 293 cells (1, 18, 25, 37, 38, 40). In addition, the utility of the AES is limited to the expression of proteins that either do not produce cytotoxic effects or interfere with AdV replication, since these situations make it impossible to generate recombinants (15). Among the genes that we were unable to rescue into AdV using the pAdBM5 transfer vector (~5% of our attempts; unpublished data) are a series of deletion mutants of the herpes simplex virus type 2 (HSV-2) ribonucleotide reductase R1 subunit. To overcome this limitation, an ideal expression cassette should be regulated in such a way that the basal level of transgene expression is low enough to avoid any interference with adenovirus replication and yet is capable of becoming very high when fully induced. Although several inducible promoters are available, the tetracycline-controllable transactivator system is becoming the most widely used in mammalian cells in cultures (11–13, 20, 24, 27, 28) and in transgenic mice (29). This system makes use of a tTA transactivator (tTA) formed by the fusion of the activation domain of HSV protein VP16 to the Escherichia coli tetracycline repressor protein (12). The tTA transactivator can stimulate transcription from a promoter containing the tetracycline operator sequences (tetO) but is prevented from interacting with tetO by tetracycline concentrations that are not toxic for eukaryotic cells (reviewed in reference 11). A modified tTA (rtTA) which interacts with tetO only when certain tetracycline analogs are present has also been developed (13). Here, we describe pAdTR5, an AdV containing a tetracycline-regulated expression cassette that inducibly overexpresses nontoxic proteins such as the HSV-2 R1 subunit at levels as high as 20 to 25% TCP in both 293 cells and nonpermissive cells. When compared with that of our newly optimized constitutive CMV-based expression cassette, pAdCMV5, the performance of pAdTR5 was found to be roughly equivalent. Moreover, we have constructed with this controllable vector a recombinant adenovirus expressing a putatively toxic R1 protein truncated at its amino-terminal end (ΔR1). tTA-expressing cells infected with this recombinant produced, before their death, ΔR1 at a level approaching 10% TCP. As the abrogation of ΔR1 synthesis with anhydrotetracycline prevented cell...
death, we could conclude that recombinant protein expression was responsible for the death process.

MATERIALS AND METHODS

Cells and viruses. The conditions for culturing of human 293 cells, either the original anchorage-dependent 293A line (16) or 293S, an anchorage-independent clone, were as described previously (9, 23). HeLa S3 and AS49 cells were obtained from the American Type Culture Collection and cultured in the same medium as that used for 293A cells. Ad5/ΔE1ΔE3 was used as the parent virus in our viral constructs (10). Viruses were purified from infected cells and titers were determined by standard protocols (23).

Construction of the transfer vectors pAdCMV5 and pAdTR5 and of recombinant adenoviruses. All recombinant DNA molecules were constructed by standard cloning and site-directed mutagenesis procedures and propagated in E. coli DH5. The transfer vector pAdCMV5 was derived from pAdCMV (rabbit β-globin)-poly(A) (18) by several subcloning steps resulting in the insertion, downstream of the +1 start site for the transcription of the human CMV IE promoter-enhancer, of a PCR fragment containing the adenovirus tripartite leader with the adenovirus major late enhancer bracketed by splice donor and acceptor sites, as found in pAdBM5 (23). In the process, an additional unique 8-bp cutter cloning site (Pmel) was added before the BamHI site (Fig. 1). pAdTR5 (tetracycline regulatable) was derived from pUHD10-3 (12) and pAdCMV5 in two steps. The same PCR fragment containing the adenovirus tripartite leader and enhancer sequences from pAdBM5 was first subcloned in pUHD10-3 downstream of the minimal CMV TATA box (PhCMV-1) of the tripartite leader and enhancer sequences (ret) replace the CAAT box and the enhancer of the CMV major IE promoter. The diagrams are not drawn to scale. SS, splicing signal; SD, splice donor; SA, splice acceptor; tpl, tripartite leader; enh, enhancer; tet, tetracycline; Ori, origin of replication; pro, promoter. The expression cassettes contain two SDs as a result of the insertion of the MLP enhancer sequence into a small intron located downstream of the tpl in pAdBM5 (19). The first SD is the one found after the second segment of the tpl whereas the second SD is the one found after the first segment of the tpl and was added when the MLP enhancer mapping at +30 to +130 relative to the MLP transcription start site was cloned as a fragment of 108 nucleotides (23).

FIG. 1. Genetic maps of pAdTR5 and pAdCMV5 transfer vectors. (A) Most of the genetic elements present in these transfer vectors have been described in detail by Massie et al. (23), and the full sequences of the plasmids are available upon request. The inner numbers on the vectors refer to map units (m.u.) on the Ad5 genome. pML2 is the E. coli replicon; the segments with dots (0 to 1 and 9.4 to 15.5 m.u.) bracketing the expression cassettes (between 1 and 9.4 m.u.) are Ad5 subgenomic portions involved in homologous recombination used to generate adenovirus recombinants. (B) Schematic representation of the expression cassettes. The expression cassettes are identical from the TATA box to the poly(A) site (the main difference between the two is the 16-bp insert upstream in pAdCMV5). The firing sequences (ret) replace the CAAT box and the enhancer of the CMV major IE promoter. The diagrams are not drawn to scale. SS, splicing signal; SD, splice donor; SA, splice acceptor; tpl, tripartite leader; enh, enhancer; tet, tetracycline; Ori, origin of replication; pro, promoter. The expression cassettes contain two SDs as a result of the insertion of the MLP enhancer sequence into a small intron located downstream of the tpl in pAdBM5 (19). The first SD is the one found after the second segment of the tpl whereas the second SD is the one found after the first segment of the tpl and was added when the MLP enhancer mapping at +30 to +130 relative to the MLP transcription start site was cloned as a fragment of 108 nucleotides (23).
cline-free medium by use of a flow cytometric β-galactosidase assay (8). A HeLa S3 cell line expressing the reverse tTA protein was established by transfection with the rtTA expression vector pUHD172-1neo followed by selection in media containing G418 (13). The resulting clones were screened by transient transfection with a tTA-regulated reporter plasmid pTR/GFP encoding the green fluorescence protein (GFP) as detailed previously (24).

Protein extraction and analysis. For analysis of recombinant protein synthesis or accumulation, petri dishes of subconfluent cells at a density of \(5.0 \times 10^5\) cells/dish were infected with viral inocula corresponding to MOIs of 10 to 20 PFU/cell for 293 cells and 50 to 800 PFU/cell for A549 or HeLa S3 cells. At different times postinfection (p.i.), total protein extracts were prepared by lysing phosphate-buffered saline-washed cells with 2% sodium dodecyl sulfate (SDS) in 80 mM Tris-HCl (pH 6.8)–10% glycerol. Before protein analysis by SDS-polyacrylamide gel electrophoresis and Western blotting, performed as described previously (19), the extracts were sonicated to shear the DNA. Quantification of the percentages of recombinant R1 and ΔR1 in total protein extracts was done by densitometric scanning of the lanes of Coomassie blue-stained gels with a Jandel Scientific video analysis system as detailed previously (23). Protein radio-labeling with \([35S]\)methionine was done as detailed previously (19).

Flow cytometry analysis of GFP expression. Exponentially growing cells (10^6) seeded in duplicate 60-mm plates were either infected with pAdSTR5GFP at increasing MOIs (A549-tTA cells) or coinfected with pAdCMVtTA at 500 PFU/cell (HeLa S3 and A549 cells). After 40 h at 37°C with or without doxycycline (1 µg/ml), the cells were fixed with 2% paraformaldehyde for 30 min at 4°C. GFP emission was analyzed with an EPICS XL-MCL flow cytofluorometer (Coulter, Miami, Fla.) equipped with a 15-mW argon ion laser and the following filters: 488-nm laser blocking, 488-nm long-pass dichroic, and 525-nm band-pass.

Ribonucleotide reductase assay. Ribonucleotide reductase activity of proteins R1 and ΔR1 was measured in crude protein extracts obtained by 2 min of sonication followed by centrifugation at 12,000 \( \times g \) for 10 min at 4°C as previously detailed (23).

**RESULTS**

Construction of the transfer vectors pAdTR5 and pAdCMV5. We have been using the AES to produce sufficient amounts of HSV-2 R1 and R2 proteins for structural studies. Although the AES was very successful for the R2 protein, the R1 protein could not be obtained with a similar yield, partly due to poor solubility (23). HSV-2 R1 is a more complex protein than cellular R1: it contains, in addition to a reductase domain, an...
assessed with the HSV-2 R1 gene by producing the adenovirus recombinants Ad5STR5R1 and Ad5CMV5R1. Our best previously produced adenovirus recombinant for the R1 gene, Ad5BM5R1, yielded in permissive 293 cells R1 levels exceeding 20% TCP (more than 60 μg/10^6 cells) (23). To complement the tetracycline-regulated expression cassette, stable clones expressing tTA or rtTA were derived from 293S (24), A549, and HeLa S3 cells as described in Materials and Methods. With a tetracycline-regulated reporter gene, the cell lines used in this study were further selected for their ability to retain, in the absence of selective pressure, both high and inducible levels of expression.

The amounts of R1 protein accumulated in nonpermissive A549-tTA and HeLa-rtTA cells at 48 h p.i. with increasing MOIs of Ad5CMV5R1 and Ad5STR5R1 are shown on Coomassie blue-stained gels in Fig. 2A and B. Ad5CMV5R1 yielded, with both cell lines, maximal levels of R1 at 800 PFU/cell (Fig. 2A and B, lanes 6), the highest MOI tested (25 to 30% TCP), and low levels of expression at an MOI of 50 (lanes 2). Somewhat higher levels of expression were achieved in HeLa-rtTA cells than in A549-tTA cells. With Ad5STR5R1, maximal levels of R1 expression were obtained at an MOI of 50 to 100 in both A549-tTA and HeLa-rtTA cells (Fig. 2A and B, lanes 7 to 11). Although the Ad5STR5R1 vector did not achieve the level of R1 production that could be obtained with the Ad5CMV5R1 vector at very high MOIs, it achieved a level of expression that approached 20% TCP. Furthermore, at lower MOIs (<50), Ad5STR5R1 was about 10-fold more efficient than Ad5CMV5R1; for example, an MOI of 5 with Ad5STR5R1 produced a substantial R1 level corresponding to 4% TCP (data not shown).

Regulation of gene expression by an AdV recombinant expressing the tTA protein. To increase the range of cell lines that could be efficiently transduced by our pAdTR5 cassette, we also constructed an AdV recombinant that could express the tTA protein under the control of the CMV promoter. In addition, as the high level of protein expression obtained in Ad5STR5R1-infected tTA cells was found to be dependent on the tTA clones used (data not shown), we hoped that higher levels of tTA could be expressed by AdV transduction than by our best stable clones and that this expression would further increase the production of R1 in Ad5STR5R1-infected cells. To determine the conditions for optimal gene expression with the tetracycline-regulatable expression system, we used AdTR5GFP, an adenovirus recombinant expressing the jelly

![FIG. 3. Constitutive or regulated overexpression of HSV-2 R1 and HSV-2ΔR1 in 293-tTA cells infected with AdV. (A) Coomassie blue-stained gel of total proteins (10 μg) extracted from 293-tTA cells uninfected (lane 1) or at 48 h p.i. with Ad5CMV5R1 (lane 2), Ad5STR5R1 (lanes 3 and 4), and Ad5STR5ΔR1 (lanes 5 and 6). The positions of the R1, hexon, and ΔR1 proteins are indicated on the right. Molecular weight markers are shown on the left in thousands. + and −, with or without anhydrotetracycline (40 ng/ml), respectively. (B) Immunoblot of the same total protein extracts probed with a rabbit polyclonal antiserum against a 19-mer peptide corresponding to amino acids 886 to 904 of HSV-2 R1 and visualized with an ECL detection kit (Amersham). The protein band comigrating with the 66-kDa marker is bovine serum albumin, which contaminated the cell extracts. Bovine serum albumin reacted strongly with this rabbit antiserum.](http://jvi.asm.org/)

### Table 1. Efficiency of gene expression regulation with the tetracycline-regulatable gene switch in AdTR5GFP-infected A549-tTA cells

<table>
<thead>
<tr>
<th>MOI</th>
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<td>0.3</td>
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*The number of transduced cells was >98% in the absence of doxycycline at every MOI. FI, fluorescence index calculated as the percentage of cells expressing GFP by the mean fluorescence value; ON, GFP expression without doxycycline; OFF, GFP expression with 1 μg of doxycycline per ml; IND, induction factor calculated as the ratio between the FI of the ON state and the basal FI; EXT, extinction factor calculated as the ratio between the FI of the ON state and the FI of the OFF state.*
lyfish GFP which is easily quantifiable by cytofluorometry (24). When A549 and HeLa S3 cells were coinfected with Ad5TR5GFP at an MOI of 50 and AdCMVtTA at increasing MOIs, we observed an increase in GFP expression that was approximately proportional to the MOI up to 500, where it plateaued (data not shown). Interestingly, infection with Ad5TR5R1 or Ad5CMVtTA alone did not produce the appearance of any new protein band detectable by Coomassie blue staining (Fig. 2C and D, lanes 3 and 4 and data not shown). This result indicated that, although being driven from a CMV-based promoter cassette, tTA was expressed at a much lower level than R1 and that R1 expression with the tetracycline-regulated promoter was very low in the absence of tTA. Altogether, these results showed not only that the tTA protein can be transduced by an adenovirus recombinant but also that in this way the transactivator appears to be produced in higher effective amounts than by the best stable cell lines.

**Overexpression of the cytotoxic ΔR1 protein.** As mentioned above, the cytotoxic nature of ΔR1 was initially suspected from our inability to generate a recombinant adenovirus expressing the deletion protein in several transfection experiments regardless of whether the pAdBM5 or pAdCMV5 vector was used. However, when the pAdTR5 vector was used, Ad5TR5ΔR1, a recombinant adenovirus expressing R1(Δ2-357), was readily obtained in the first attempt. An initial analysis of ΔR1 expression in permissive 293-tTA cells infected with Ad5TR5ΔR1 for 48 h indicated that the level of ΔR1 accumulation was lower (10% TCP) than that of the full-length R1 in cells infected with either Ad5CMV5R1 or Ad5TR5R1 (20 to 25% TCP) (Fig. 3, compare lane 6 with lanes 2 and 4). Moreover, whereas in cells infected with Ad5TR5ΔR1 the accumulation of several viral polypeptides, including the hexon and 100K protein, was severely reduced (Fig. 3, compare lanes

![FIG. 4. Cytotoxicity of HSV-2 ΔR1. A549-tTA cells (10^5 in six-well plates) were infected 24 h after seeding with 100 PFU of Ad5TR5R1 ( ), Ad5TR5ΔR1 ( ), or Ad5TR5ΔR1 plus 30 ng of anhydrotetracycline per ml ( ) per cell or were mock infected ( ).](http://jvi.asm.org/)

![FIG. 5. Time course of HSV-2 R1 and HSV-2 ΔR1 synthesis in A549-tTA cells infected with tetracycline-regulatable Adv. A549-tTA cells were either uninfected (lane 1) or infected with Ad5TR5R1 (lanes 2 to 5) or Ad5TR5ΔR1 (lanes 6 to 9) at an MOI of 100, and total proteins were extracted following 2 h of radiolabeling with [35S]methionine at 2 h (lanes 2 and 6), 6 h (lanes 3 and 7), or 24 h (lanes 4, 5, 8, and 9) p.i. Autoradiography was performed on a gel loaded with 10 μg of protein per lane. The positions of the R1 and ΔR1 proteins are indicated on the right. + and −, with or without anhydrotetracycline (40 ng/ml).](http://jvi.asm.org/)
R1 and R1 at an MOI of 100 (Fig. 5). The synthesis of both Ad5TR5R1 (Fig. 3, compare lanes 2, 3, and 4). The reduction in the synthesis of viral polypeptides might explain why we were unable to generate a recombinant virus for ΔR1 with a constitutive expression cassette. Consistent with this idea, the titers of Ad5TR5AR1 grown on 293-TA cells under induced conditions (in the absence of anhydrotetracycline) was 20-fold lower than when the expression of ΔR1 was repressed (data not shown). The experiment presented in Fig. 3 also shows that the expression of R1 or ΔR1 could be repressed about 25-fold by the addition of 40 ng of anhydrotetracycline per ml (compare lanes 3 and 5 with lanes 4 and 6). Repression was not complete, likely due to low-level transactivation of the minimal TATA box by adenovirus E1 proteins (17, 39) in combination with the high copy number of the viral vector following replication in permissive 293 cells. The extent of repression that could be achieved by this system was quantified more precisely with Ad5TR5GF and nonpermissive A549-tTA cells. As can be seen in Table 1, it was greater than 50-fold at MOIs of 50 to 250 PFU/cell. Interestingly, at the highest MOI, the extinction factor was decreased to a value of 28, which corresponds to the repression observed in permissive 293 cells. The nearly linear relationship between the MOI of Ad5TR5GF and the basal expression level indicates that the copy number of the tetracycline-regulated expression cassette is the most important factor which determines the basal expression level (Table 1).

To avoid the cytopathic effects associated with adenovirus replication, we used nonpermissive cells to further examine the cytotoxic nature of the ΔR1 protein. The marked cytotoxicity of ΔR1 in A549-TA and HeLa-rtTA cells infected with Ad5TR5AR1 was evidenced by drastic morphological cellular changes and a gradual decline in attached cell numbers, as exemplified in Fig. 4 for A549-TA cells. The first signs of a cytopathic effect could be seen by 5 h p.i., when cytoplasmic blebbing appeared in about 5% of the Ad5TR5AR1-infected cells. Blebbing was soon followed by cell rounding and detachment. By 12 h p.i., approximately 15 to 20% of the Ad5TR5AR1-infected cells appeared to be in the medium (Fig. 4A, bottom left panel). By 48 h p.i., about half of the input cells remained attached to the culture flask, and these cells were enlarged in size and granular in appearance (Fig. 4A, bottom right panel). There was no apparent sign of cytotoxicity in Ad5TR5R1-infected cells (Fig. 4A, top panels) or in cells infected with Ad5TR5AR1 in the presence of anhydrotetracycline (data not shown). Whereas cell numbers increased in both mock-infected cells and cells infected with Ad5TR5R1, proliferation of the Ad5TR5AR1-infected cells did not occur and the numbers of attached cells gradually declined to a level, at 55 h p.i., approximately 60% of the starting cell density (Fig. 4B). Cells infected with Ad5TR5ΔR1 in the presence of anhydrotetracycline initially grew, and after 30 h, the cell numbers plateaued in a manner similar to that of Ad5TR5R1-infected cells (Fig. 4B), demonstrating that the accumulation of the ΔR1 protein brings about the demise of Ad5TR5AR1-infected cells.

In infected A549-TA cells, the ΔR1 protein accumulated to a level of about 10% TCP, like 293-TA cells (data not shown). To determine whether the lower level of ΔR1 accumulation was related directly to a lower rate of synthesis or indirectly to its cell-killing effect, we analyzed the rates of synthesis of R1 and ΔR1 at various times p.i. by [35S]methionine pulse-labeling of A549-TA cells infected with either Ad5TR5R1 or Ad5TR5AR1 at an MOI of 100 (Fig. 5). The synthesis of both R1 and ΔR1 proteins, which were detectable in total cell extracts as early as 2 h p.i., occurred at similar rates until 6 h. However, between 6 and 24 h p.i., the synthesis of the full-length R1 protein exceeded that of the ΔR1 protein (Fig. 5, compare lanes 3 and 4 with lanes 7 and 8). Beyond 24 h, the synthesis of the ΔR1 protein as well as other cellular proteins decreased dramatically, whereas in Ad5TR5R1-infected cells, the synthesis of the R1 protein remained elevated, like general protein synthesis, up to 72 h p.i. (data not shown). General protein synthesis was also not impaired in the presence of anhydrotetracycline at a dose that completely repressed ΔR1 protein synthesis in A549-tTA cells infected with Ad5TR5ΔR1 (Fig. 5, lane 9) or R1 protein synthesis in Ad5TR5R1-infected cells (Fig. 5, lane 5). As the initial rates of synthesis of both proteins were similar, it appears more likely that the lower level of ΔR1 accumulation was an indirect effect of its toxicity.

Lastly, we examined if our initial objective of obtaining a more soluble protein had been attained by evaluating the solubility of R1 and ΔR1 proteins accumulated in total cell extracts. Unfortunately, the ΔR1 protein was somewhat less soluble (15% solubility) than our reference full-length R1 protein produced in 293 cells infected with Ad5BM5R1 (30% solubility). Nevertheless, the intrinsic activity of the ΔR1 protein present in the soluble fraction, as measured by an in vitro ribonucleotide reductase assay, was similar to that of the R1 protein, indicating that the first 357 amino acids of the protein were not essential to its reductase activity (data not shown).

Interestingly, the full-length R1 protein produced in A549-TA cells infected with Ad5TR5R1 exhibited nearly twofold more solubility than our reference R1 protein.

**DISCUSSION**

The pAdTR5 and pAdCMV5 transfer vectors significantly improved the utility of AdV for high-level transgene expression in mammalian cells. First, each of these vectors allowed for very high levels of recombinant protein production in nonpermissive cell lines. This fact greatly simplified the production and purification effort, since the recombinant protein could be produced at levels approaching 15 to 25% TCP in the absence of the synthesis of other viral proteins. Second, by incorporation of the tetracycline-regulated promoter into the expression vector, it was possible to use the AES to produce toxic proteins that interfered with adenovirus replication at levels approaching those which can be achieved by use of a constitutive expression cassette.

In comparison with the pAdTR5 and pAdCMV5 transfer vectors, adenovirus MLP-based expression cassettes such as pAdBM5-derived AdV, which yielded the best protein production levels in permissive 293 cells (23), typically yielded, at equivalent MOIs, three- to fivefold-lower expression levels in nonpermissive cells, depending on the transgene tested (unpublished results). This observation is consistent with previous reports showing that adenovirus MLP-based expression cassettes, even when modified with additional enhancer sequences, are less efficient than CMV promoter-based expression cassettes in most nonpermissive cell lines (38, 40). Although the relative contributions of the various elements cloned downstream of the CMV TATA box to the improvement in transgene expression in AdV were not systematically assessed, we suspect that in nonpermissive cells the most significant cis-acting elements are the adenovirus tripartite leader and splice sites. In fact, the adenovirus major late enhancer is not likely to be active in the absence of viral protein IVa2, which is normally expressed after DNA replication begins (21); even at a high MOI in A549 or HeLa cells, we did not find evidence that the adenovirus late proteins were expressed at
significant levels, suggesting that levels of IVa2 were minimal. Nevertheless, the adenovirus major late enhancer was included in the expression cassette both to take advantage of its activity in permissive 293 cells and also to obtain a direct comparison with pAdBM5, our best-characterized transfer vector (23).

The high level of expression attained in nonpermissive cell lines with our new vectors is substantially greater than the previously reported expression levels of about 2% TCP obtained with nonoptimized CMV promoter-based expression cassettes (1, 18, 25, 37, 38, 40; unpublished results). The exception is a study by Wilkinson and Akirig in which an expression level in the range of 20% TCP was obtained in MRC5 cells infected with an AdV expressing the E. coli lacZ gene (37). However, this high level of expression of β-galactosidase required the addition of forskolin, which stimulated the CMV promoter more than 10-fold. Furthermore, overexpression of β-galactosidase was obtained only after 144 h p.i.; at 48 h p.i., a time corresponding to the peak of expression with our optimized expression cassettes, the expression of β-galactosidase was fivefold lower.

We used the tetracycline-regulatable adenovirus cassette to produce, at high levels, a protein that was suspected to be cytotoxic, and we confirmed the cytotoxic nature of the ΔR1 protein by showing that infection of AS49-tTA cells with the AdSTR5ΔR1 vector brought about an eventual demise of the infected cells. The cytotoxicity of the ΔR1 protein was totally unexpected. Although we do not have any clues to explain it at this point, it is evident that overexpression per se was not the cause, since the R1 protein was expressed at even higher levels. Furthermore, preliminary experiments indicated that cytotoxicity could be observed at levels lower than 0.01% TCP (19a). This cytotoxic effect and the synthesis of the ΔR1 protein could be nearly completely abrogated by inhibition of the activity of the tTA protein with anhydrotetracycline in Ad5STR5ΔR1-infected cells.

We have also generated a number of other recombinant adenoviruses using the pAdTR5 vector. The vector has made it possible to overexpress, at more than 10% TCP, the human enzyme methenyltetrahydrofolate synthetase, the heat-inducible chaperone protein hsp70, the adenovirus E1B-19K protein, and the viral protein encoded by ORF5 of the porcine reproductive and respiratory syndrome virus (unpublished data). This last protein was recently shown to induce apoptosis when expressed in BSC40 cells with the T7 vaccinia virus system (32). We have also shown that the expression of ΔR1 by infection with AdSTR5ΔR1 induces the death of AS49 and HeLa S3 cells by an apoptotic process (19a). The characterization of the apoptotic behavior of ΔR1 or any other proteins with similar behavior will be facilitated by the use of an expression system allowing for the tight regulation of gene expression in the absence of any other toxic effects resulting from the infection of permissive cells, such as is the case with vaccinia virus vectors. Curiously, the adenovirus E1B-19K protein, which exhibited potent antiapoptotic activity in both adenovirus-infected cells and stable cell lines (35; reviewed in reference 33), could be overexpressed only with the pAdTR5 vector. We found that at high MOIs in nonpermissive cells, the overexpression of E1B-19K (≥2% TCP) was very toxic, killing the cells by necrosis (unpublished results).

Another elegant molecular switch, based on the Cre-loxP recombination system, has been described for the regulation of gene expression in AdV (3). With this system, the transgene is placed under the control of a strong constitutive promoter, such as the CMV promoter, and its expression is silenced by the inclusion of a spacer fragment of 1.3 kbp between the promoter and the open reading frame. This spacer fragment is flanked by two lox sites, upon which the Cre recombinase acts to precisely excise it, thereby restoring efficient expression of the transgene. Although this vector system has the potential of expressing toxic gene products in AdV, overexpression of toxic proteins has not been reported thus far with it. Furthermore, the Cre-loxP system is less flexible than the tetracycline-regulated gene switch described here in two respects. First, the need for a spacer fragment larger than 1 kbp limits the available space for the cloning of transgenes into AdV, thereby reducing the option for cloning large cDNAs or constructing AdV with double expression cassettes (6). Second, since it is not possible to regulate the Cre recombinase switch with chemical inducers, one is restricted to the scenario of coinfection with two viruses instead of being able to combine the transactivator with the inducible expression cassette in one virus, as we are currently doing with the tetracycline-regulated gene switch. This latter approach was reported to be more efficient for the generation of transgenic animals with regulated transgene expression (29) and should prove to be equally efficient for gene transfer experiments with AdV.

Finally, in addition to the application of the AES to protein production in animal cell cultures, the pAdCMV5 and pAdSTR5 vectors will be useful for functional studies and gene therapy applications. Adenoviruses are already well established as effective vectors for human gene therapy applications. The higher level of expression that can be achieved with these AdV in nonpermissive cells will make it possible to express transgenes at significant levels in vivo at lower MOIs, thereby allowing minimization of the toxic side effects associated with the load of adenovirus particles. For example, Connolly et al. recently reported that a threefold improvement in the level of expression of blood coagulation factor VIII following AdV transduction allowed for the injection of significantly lower vector doses while still providing factor VIII at therapeutic levels (7). Furthermore, the tTA system has been shown to be highly effective for the regulated expression of recombinant proteins in transgenic animals (29). The tetracycline-regulated AES that we have described here will make it possible to express in vivo by coinfection with an AdV expressing tTA a therapeutic protein at levels that can be precisely regulated by the in vivo concentration of the effector molecule tetracycline. We are currently assessing the usefulness of such an inducible vector for in vivo gene transfer experiments.

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