NOTES

Diminishing Adenovirus Gene Expression and Viral Replication by Promoter Replacement

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The adenovirus E4 promoter was replaced by a synthetic promoter composed of a minimal TATA box and five consensus 17-mer yeast GAL4-binding-site elements. The viral vectors, which also contained human factor IX (hFIX) cDNA driven by Rous sarcoma virus long terminal repeat in the E1 region, were then constructed and expanded in 293 cells permanently expressing GAL4/VP16 fusion protein. Viral replication and expression of adenovirus E4 genes and late genes (hexon and fiber) were evaluated in vitro in the human lung carcinoma cell line H1299. Viral replication and viral gene expression were dramatically reduced in the cells transduced by vectors with a replaced E4 promoter compared to the levels in the cells transduced by vectors with the wild-type E4 promoter. The levels of transgene (hFIX) expression remained similar between vectors with or without E4 promoter replacement. These results indicate that diminution of viral gene expression and viral replication is achievable by promoter replacement.

Diminution of viral gene expression and viral replication is desirable for the development of attenuated live viral vaccines and viral vectors used for gene therapy. For example, inactivation of a cytotoxic viral gene could reduce the virulence of a virus sufficiently to allow the production of attenuated live viral vaccines. Diminishing viral gene expression in transduced cells is believed to reduce vector immunogenicity following virally mediated gene therapy, thus increasing the survival of transduced cells and resulting in prolonged transgene expression. This may result in an increase in the safety of viral vectors, allowing doses to be raised to levels necessary to achieve the desired biological or therapeutic effects.

Viral gene expression can be diminished via gene mutations or deletions. Mutagenesis in particular can be achieved by using various reagents and procedures. Viral mutants can then be selected in accordance with changes in growth properties, such as temperature sensitivity or host range restrictions (3, 6). Random mutagenesis, however, can result in the accumulation of many mutations, making it difficult to predict their functional consequences. Deletion of partial or entire coding regions of a viral gene will ultimately result in abrogation of viral gene expression. However, the required functions of the deleted viral gene must be supplied in *trans* for viral propagation. Consequently, a packaging cell line transformed with the deleted gene is usually required for efficient viral propagation (7, 20, 21). Unfortunately, establishing a cell line that contains several viral genes usually proves to be a most difficult and time-consuming task because the deleted genes are frequently toxic to host cells.

Theoretically, viral gene expression could be diminished by switching off the control elements, or promoters, of a gene.

Expression of viral genes in packaging cells but not in vivo in humans could be achieved by replacing the viral gene promoter with an inducible or synthetic promoter that is active in the packaging cell lines only when a specific transactivating protein is provided. To test the feasibility of inactivating viral gene expression and viral replication by promoter replacement, we used a synthetic promoter composed of a minimal TATA box and five consensus 17-mer yeast GAL4-binding-site elements (16). Since most mammalian cells express no GAL4-like activity, a synthetic GAL4-responsive promoter containing GAL4-binding sites and a TATA box should have extremely low basal activity in the absence of a GAL4 transactivator and high inducibility in its presence (2, 16). We used the adenovirus E4 region as a model. The adenovirus E4 transcription unit lies between map units 91 and 100 and encodes a variety of different mRNAs required for efficient DNA replication, late-gene expression, and host gene shutoff (8). Mutants lacking most of the E4 region are severely replication defective and can be propagated only in cell lines containing and expressing E4 (8, 13, 20, 21). Here we describe the construction and in vitro evaluation of adenovirus vectors whose E4 promoter was replaced by GAL4/TATA. We also show that diminishing viral gene expression and viral replication are indeed achievable through promoter replacement.

293 cells transformed with GAL4/VP16. A 293 cell line expressing GAL4 transactivating protein is required for generating an adenovirus vector whose E4 promoter is replaced by GAL4/TATA. Naive 293 cells were transfected by the $\text{Ca}_2(\text{PO}_4)_2$ method with pM2/VP16 (16) (a gift of I. Sadowski, University of British Columbia, Vancouver, Canada) and pRSV/Neo, which encodes a neomycin resistance gene driven by a Rous sarcoma virus long terminal repeat (RSV LTR), at a pM2/VP16:pRSV/Neo ratio of 10:1. The G418-resistant colonies were isolated and tested for their ability to transactivate GAL4/TATA promoter in plasmid pG5EC (16) (also the gift of I. Sadowski). Of 20 colonies, 3 were capable of activating GAL4/TATA (data not shown). The duplication time, trans-

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FIG. 1. Construction and characterization of adenovirus vectors containing RSV-hFIX. (a) Schematics of the two plasmids used to construct adenovirus vectors whose E4 promoter was replaced by GAL4/TATA. The open bars represent adenovirus sequences; the numbers indicate the corresponding positions in the viral genome. Solid areas represent a minimal promoter consisting of five consensus 17-mer GAL4-binding sites upstream from the adenovirus E1B TATA box. (b) Schematics of the three recombinant adenovirus constructs containing the RSV-hFIX expression cassette. The expression cassette, containing an RSV LTR, hFIX cDNA, and a bovine growth hormone polyadenylation signal sequence in a right-to-left orientation, was placed in the adenovirus E1 region. AdE4A/RSV-hFIX and AdE4B/RSV-hFIX were constructed from pBF7A and pBF7B, respectively. Solid boxes represent the GAL4/TATA promoter.

fection efficiency, and viral production efficiency in these cells remained the same as in their parental 293 cells. One of these transformed cells (GV16) was then used to construct adenovirus vectors with E4 driven by GAL4/TATA.

Adenovirus vector with an E4 region driven by the GAL4/TATA promoter. We initially attempted to construct a vector in which GAL4/TATA would drive an E4 expression cassette inserted into the E1 region of the *dl366* backbone (8). This failed, however, because all of our viral preparations were contaminated with wild-type E1 or E4 virus. Several rounds of plaque purification did not solve the problem. We attributed this failure to the instability of the viral constructs, presumably due to the overlapping of E4 sequences in the E1 region with those in the *dl366* backbone. In a second attempt, we constructed vectors by replacing the E4 promoter with GAL4/TATA in the original E4 region. In brief, two transfer plasmids (pBF7A and pBF7B) were constructed on a pNEB193 backbone (New England Biolabs, Beverly, Mass.). Both contained adenovirus sequences from bp 25292 to the right end of viral genome (Fig. 1a). pBF7A has a deletion from bp 35575 to 35786, while pBF7B has a deletion from bp 35575 to 35818. These deletions span the E4 promoter region from +35 to -176 and from +35 to -208, respectively, and should have largely abolished the promoter activity of the E4 transcriptions (9, 10). A 143-bp fragment from pG5EC consisting of five consensus 17-mer GAL4-binding sites and a TATA box (16) was used to fill in the deleted region in the same orientation as native E4 promoter. A recombinant adenovirus vector containing human factor IX (hFIX) cDNA driven by the RSV LTR, Ad/RSV-hFIX, was constructed by a method described previously (4). AdE4A/RSV-hFIX and AdE4B/RSV-hFIX were constructed by cotransfection of 293/GV16 cells with a 27-kb *SrfI* fragment from Ad/RSV-hFIX and either pBF7A (for AdE4A/RSV-hFIX) or pBF7B (for AdE4B/RSV-hFIX). All constructs were plaque purified. The structures of these vector constructs (Fig. 1b) were confirmed by DNA analysis and by a

functional assay for the presence of hFIX in the medium of the cultured cells transduced with the constructs.

Single plaques from each of the constructs were then expanded in 293/GV16 cells and purified through two cycles of CsCl gradient ultracentrifugation. Virus titers were measured by optical absorbance ($1 A_{260}$ unit = 10^{12} particles/ml) and by plaque assay on cultured 293/GV16 and parental 293 cells. The viral concentration and total yield were similar for AdE4B/RSV-hFIX and Ad/RSV-hFIX, about twice as much as in AdE4A/RSV-hFIX. Plaque assays showed similar numbers of PFU in 293 and 293/GV16 cells for Ad/RSV-hFIX, while the numbers of PFU were 10-fold lower in 293 cells than in 293/GV16 cells for both AdE4A/RSV-hFIX and AdE4B/RSV-hFIX (data not shown). The PFU obtained in 293/GV16 cells were used in the subsequent experiments. All viral preparations were negative for contamination by the E1⁺ virus when assayed by a PCR-based technique (5). AdE4A/RSV-hFIX and AdE4B/RSV-hFIX preparations were negative for E4 wild-type virus contamination when examined by PCR with a pair of primers that amplify adenovirus sequences stretching from bp 35460 to 35935 (data not shown).

Propagation of the recombinant adenoviruses. The efficiencies of viral propagation in 293 cells and 293/GV16 cells were compared by analyzing viral growth in the two cell lines. The growth curve of Ad/RSV-hFIX was the same in both 293 cells and 293/GV16 cells, with a peak value of 2,000 to 4,000 infectious units/cell being reached between days 2 and 4. The growth curve of AdE4A/RSV-hFIX and AdE4B/RSV-hFIX in 293/GV16 cells mimicked that of Ad/RSV-hFIX, with the peak value of 1,000 to 4,000 infectious units/cell being reached on day 3. The peak values of AdE4A/RSV-hFIX and AdE4B/RSV-hFIX in 293 cells were typically 1 to 2 orders of magnitude lower than that in the 293/GV16 cells (data not shown). These results correlated with those of plaque assays, where the PFU for both AdE4A/RSV-hFIX and AdE4B/RSV-hFIX were reduced about 10-fold in naive 293 cells from the values

FIG. 2. Propagation of adenovirus vectors in H1299 cells. (a) Virus recovered from infected cells. The initial inoculation doses are indicated above each graph. The infectious units recovered were determined by the 50% tissue culture infective dose assay and are presented as $\log_{10}$ total infectious units/ 10^6 cells. The values represent the mean of duplicate assays. ○, Ad/RSV-hFIX; △, AdE4A/RSV-hFIX; □, AdE4B/RSV-hFIX. (b) Southern blot analysis of viral DNA in infected H1299 cells. Cellular DNA (5 μ g) was digested with *Cla*I and fractionated on 1% agarose gel. The viral constructs, hours postinfection, and MOI used for infection are indicated above each lane.

in 293/GV16 cells. Together, these results suggested that the E4 functions of the two viral constructs with the promoter replacement were impaired but not completely abolished in the naive 293 cells. The leakage of GAL4/TATA in naive 293 cells was not unexpected, because E1A proteins are known to be able to activate transcription through the TATA motif (18, 22), partly due to the ability of the 13S E1A protein to bind specifically and stably to the TATA box-binding protein (11, 14).

Adenovirus replication in non-E1-transformed cells was tested in a human lung carcinoma cell line (H1299). Cells (10^6 /well) were seeded in six-well plates and then infected with the various recombinant viruses at a multiplicity of infection (MOI) of 10 and 100. The cells were harvested at 2, 48, and 96 h after viral inoculation. The cell lysate was subjected to titer determination in 293/GV16 cells by determining the 50% tissue culture infective dose of the virus (12). At an MOI of 10, the total number of infectious units remained unchanged from 2 to 96 h postinfection in Ad/RSV-hFIX-infected cells. At the same MOI, the total number of infectious units dropped from about 10^3 to undetectable levels for both AdE4A/RSV-hFIX- and AdE4B/RSV-hFIX-infected cells (Fig. 2a). At an MOI of 100, a significant increase in the number of infectious units was observed in cells infected with Ad/RSV-hFIX between 2 and 96 h postinfection. Meanwhile, at the same high MOI, the total number of infectious units remained unchanged during the period of study in the cells infected with either AdE4A/RSV-hFIX or AdE4B/RSV-hFIX (Fig. 2a). These results were further supported by the analysis of DNA from the infected cells.

FIG. 3. Northern blot analysis of viral gene expression. Total RNA (20 μ g) from transduced cells was loaded on a 1.2% agarose gel. Lanes: 0, mock infection; W, Ad/RSV-hFIX; A, AdE4A/RSV-hFIX; B, AdE4B/RSV-hFIX. The numbers below W, A, and B represent MOIs.

FIG. 4. Hexon gene expression in cells transduced with adenovirus vectors. H1299 (a to c) and A549 cells (d to f) were transduced with Ad/RSV-hFIX (a and d), AdE4A/RSV-hFIX (b and e), or AdE4B/RSV-hFIX (c and f) at an MOI of 100. The cells were then incubated at 37°C for 48 h. Hexons (green) were visualized with FITC-labeled polyclonal goat antibody. The photographs are representative of two duplicate experiments.

Southern blot analysis showed a dramatic increase in viral DNA in the cells infected with Ad/RSV-hFIX at an MOI of 100 between 2 and 96 h after viral infection (Fig. 2b). At the same high MOI, the increase in the level of viral DNA was greatly retarded in the cells infected with AdE4A/RSV-hFIX and AdE4B/RSV-hFIX. Southern blot and PCR analysis of the DNA from cells 96 h after infection with all three RSV-hFIX-containing viral constructs excluded the presence of E1⁺ virus (data not shown), indicating that E1⁺ virus contamination probably did not contribute to viral replication in H1299 cells infected with Ad/RSV-hFIX. These results correlated well with previous observations by others that at a high MOI, E1-deleted adenovirus can replicate in non-E1-complementing cells (17). These results also demonstrated that replacement of the E4

promoter with GAL/TATA further diminished viral replication in non-E1-transformed cells.

Viral gene expression in cultured human carcinoma cell lines. To test E4 expression in the E1⁻ cells, we infected H1299 cells with the three RSV-hFIX-containing viral constructs at MOIs of 10 and 100. Mock-infected H1299 cells were used as negative controls, while 293/GV16 cells infected at an MOI of 2 were used as positive controls. Preliminary experiments showed that over 80% of the H1299 cells were transduced at an MOI of 10. The cells were harvested 2 days after infection, and total RNA was isolated. Northern blot analysis showed that the E4 transcriptions in all the constructs tested were greatly reduced in H1299 cells compared with 293/GV16 cells (Fig. 3), supporting the role of E1 in regulating E4 func-

TABLE 1. hFIX in the media of cultured cells transduced with recombinant adenovirus vectors containing RSV-hFIX

Cell line	Vector	MOI	Level of hFIX (ng) ^a
H1299	Ad/RSV-hFIX	10	169.9 ± 7.6
	AdE4A/RSV-hFIX	10	224.2 ± 27.1
	AdE4B/RSV-hFIX	10	116.4 ± 17.6
	Ad/CMV-LacZ	10	0
	Ad/RSV-hFIX	100	293.4 ± 45.3
	AdE4A/RSV-hFIX	100	298.9 ± 46.4
	AdE4B/RSV-hFIX	100	301.1 ± 44.3
	Ad/CMV-LacZ	100	0
A549	Ad/RSV-hFIX	100	136.2 ± 45.7
	AdE4A/RSV-hFIX	100	67.9 ± 4.7
	AdE4B/RSV-hFIX	100	61.55 ± 9.0
	Ad/CMV-LacZ	100	0

^a hFIX levels represent mean ± standard deviation in duplicate experiments; they are presented as the total hFIX yields from 10⁵ cells after 48 h of incubation at the indicated MOIs.

tion (1, 15, 17). Nevertheless, at the high MOI (MOI = 100), E4 transcription was quite substantial in H1299 cells infected with Ad/RSV-hFIX. In contrast, at the same high MOI, E4 transcription was dramatically reduced in the H1299 cells infected by viral constructs whose E4 promoter was replaced by GAL4/TATA, indicating that GAL4/TATA has much less transcriptional activity in non-E1-transformed cells than does the wild-type E4 promoter.

The adenovirus E4 region encodes proteins required for late-gene expression. Transcription rates for late genes in the cells infected with E4-deleted virus were reduced to 10% of those for late genes in the cells infected with the wild type (8). Thus, diminution of E4 function by promoter replacement should reduce viral late-gene expression. Expression of the viral fiber gene in H1299 cells was analyzed in the same Northern blot used to detect E4 mRNA (Fig. 3). Such expression correlated with that of E4. Moreover, a substantial amount of fiber transcript was detected only in H1299 cells infected by the vector with a wild-type E4 promoter at an MOI of 100. In contrast, fiber expression in H1299 cells infected by the vectors with a replaced E4 promoter was greatly diminished compared with that in cells infected by vectors with wild-type E4.

Hexon gene expression from the three RSV-hFIX-containing vectors was determined in two human lung carcinoma cell lines, H1299 and A549. The cells were infected at MOIs of 0, 10, and 100 and then cultured at 37°C for 2 days. Hexon protein was detected by staining with a fluorescein isothiocyanate (FITC)-labeled antibody that specifically recognizes the adenovirus hexon. At an MOI of 10, about 10% of H1299 cells and less than 1% of A549 cells infected with Ad/RSV-hFIX were FITC positive, consistent with our previous observation that adenovirus-mediated transduction efficiency is higher in H1299 cells than in A549 cells. In comparison, neither cell line was FITC positive when transduced with AdE4A/RSV-hFIX or AdE4B/RSV-hFIX at an MOI of 10 (data not shown). At an MOI of 100, about 50% of H1299 cells and 10 to 25% of A549 cells infected with Ad/RSV-hFIX were FITC positive. In comparison, only 1 to 5% of H1299 cells were positive when infected with AdE4A/RSV-hFIX or AdE4B/RSV-hFIX, while A549 cells infected with AdE4A/RSV-hFIX or AdE4B/RSV-hFIX remained FITC negative (Fig. 4). To test whether viral gene expression in the new vectors would increase to that of wild-type E4 in prolonged cultures, hexon gene expression in H1299 cells was analyzed 7 days after infection. Expression of

the hexon should also reflect the levels of viral genome replication required for late-gene expression (8, 19). No difference in FITC labeling was found between days 2 and 7 in the cells infected with either AdE4A/RSV-hFIX or AdE4B/RSV-hFIX at an MOI of 10 or 100. In contrast, a dramatic increase in FITC labeling was found on day 7 in the cells infected with Ad/RSV-hFIX at an MOI of 10. Curiously, no significant increase in FITC labeling was found in the cells infected with Ad/RSV-hFIX at an MOI of 100. However, a dramatic cell loss and an obvious cytopathological change were observed on day 7 in this group only (data not shown). We assume that most of the lost cells were FITC positive. These results demonstrate that diminished viral gene expression and viral replication in the new vectors do not increase to the levels achieved by E4 wild-type vectors during prolonged culture.

Expression of transgenes. Levels of hFIX in the culture media of infected cells reflected the efficiency of gene delivery by the vectors. These levels were determined by enzyme-linked immunosorbent assay (Table 1). No detectable hFIX was found in media from mock-infected cells or from cells infected with Ad/CMV-LacZ. hFIX levels in media from A549 cells infected with Ad/RSV-hFIX were about twice those in media from cells infected with AdE4A/RSV-hFIX or AdE4B/RSV-hFIX at an MOI of 100. The significance of the difference, however, is not yet clear. At an MOI of 10, hFIX was undetectable in the media from A549 cells infected with any of the three RSV-hFIX-containing constructs. In contrast, hFIX levels in the media from H1299 cells infected with the same three RSV-hFIX-containing constructs were equivalent at all MOIs tested. These results demonstrated that the transduction efficiencies of the three RSV-hFIX-containing vectors were similar in the two human cell lines tested, indicating in turn that reduction of viral gene expression in cells transduced with AdE4A/RSV-hFIX or AdE4B/RSV-hFIX was not caused by decreased transduction efficiencies. Together, our results suggest that the promoter replacement strategy might provide an alternative approach for developing viral vectors for gene therapy. Moreover, the same strategy might also be applied to developing an attenuated live viral vaccine.

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