Cytotechnology **28:** 53–64, 1998.

New adenovirus vectors for protein production and gene transfer

Bernard Massie[1*], Dick D. Mosser[1], Maria Koutroumanis[1], Isabelle Vitté-Mony[1†], Linda Lamoureux[1], France Couture[1‡], Luc Paquet[1§], Claire Guilbault[1,2], Julie Dionne[1], Dounia Chahla[1], Pierre Jolicoeur[1¶] & Yves Langelier[2]

[1] *Institut de Recherches en Biotechnologie, 6100 Royalmount Avenue, Montréal, Québec, H4P 2R2 Canada*
E-mail: bernard.massie@nrc.ca

[2] *Institut du Cancer de Montréal, Centre de Recherche Louis Charles Simard, 1560 Sherbrooke Est, Montréal, Québec, H2L 4M1 Canada*

Received 25 August 1998; accepted 25 August 1998

Key words: adenoviral recombinant, functional genomics, gene therapy, green fluorescent protein, inducible gene expression, protein production

Abstract

Based on two new adenovirus expression cassettes, we have constructed a series of Ad transfer vectors for the over-expression of one or two genes either in a dicistronic configuration or with separate expression cassettes. Inclusion of the green or blue fluorescent protein in the vectors accelerates the generation of adenovirus recombinants and facilitates the functional characterization of genes both *in vitro* and *in vivo* by allowing easy quantification of gene transfer and expression. With our optimized tetracycline-regulated promoter (TR5) we have generated recombinant adenoviruses expressing proteins, that are either cytotoxic or which interfere with adenovirus replication, at levels of 10–15% of total cell protein. Proteins that are not cytotoxic can be produced at levels greater than 20% of total cell protein. As well, these levels of protein production can be achieved with or without adenovirus replication. This yield is similar to what can be obtained with our optimized human cytomegalovirus-immediate early promoter-enhancer (CMV5) for constitutive protein expression in non-complementing cell lines. Using the green fluorescent protein as a reporter, we have shown that a pAdCMV5-derived adenovirus vector expresses about 6-fold more protein in complementing 293 cells and about 12-fold more in non-complementing HeLa cells than an adenovirus vector containing the standard cytomegalovirus promoter. Moreover, a red-shifted variant of green fluorescent protein incorporated in one series of vectors was 12-fold more fluorescent than the S65T mutant, making the detection of the reporter protein possible at much lower levels of expression.

Abbreviations: Ad – adenovirus; AdV – adenovirus vector; AES - adenovirus expression system; CMV-IE – cytomegalovirus immediate early; GFP/BFP – green/blue fluorescent protein; MLP – major late promoter; MOI – multiplicity of infection; TCP – total cell protein; tTA - tetracycline-controlled transactivator; rtTA – reverse tTA.

Introduction

The adenovirus expression system (AES) is an efficient tool both for high level protein production and for gene transfer experiments. Recombinant adenovirus vectors (AdV) can be produced at high titers, infect a wide variety of cell types both *in vitro* and *in vivo*, including non-replicating cells, and can permit high levels of gene expression (reviewed in: Acsadi et

* Author for all correspondence. Other affiliations are: Centre de Recherche en Virologie, Institut Armand-Frappier, Université du Québec, Laval, Québec, Canada H7N 4Z3; Département de Microbiologie et Immunologie, Université de Montréal, Montréal, Québec, Canada H3C 3J7

† Present address: Diagnocure Inc., Québec, Québec Canada

‡ Present address: Centre de Recherche Hospitalier de l'Université Laval, Québec, Québec, Canada

§ Present address: Exogen Neuroscience Inc., Montréal, Québec, Canada

¶ Present address: Biophage Inc., 6100 Royalmount Avenue, Montréal, Québec, H4P 2R2 Canada

al., 1995; Berkner, 1988, 1992; Graham and Prevec, 1992; Stratford-Perricaudet and Perricaudet, 1994; Trapnell and Gorzilla, 1994). Recent improvements to the expression cassette in the transfer vector (pAdCMV5) have resulted in attaining expression levels greater than 20% total cell protein (TCP) in 293 cells and in non-permissive cells (Massie et al., 1998). The inability to generate AdV expressing proteins that have cytotoxic effects or which interfere with AdV replication has been overcome by the development of a transfer vector (pAdTR5) that utilizes a tetracylcine-regulated expression cassette (Massie et al., 1998). A similar vector was also recently described by Hu et al. (1997). Infection of cells that express the tetracycline-controlled transactivator tTA (Gossen and Bujard, 1992) or rtTA (Gossen et al., 1995) or co-infection together with an AdV that expresses tTA can allow production of toxic proteins at levels approaching 10% TCP (Massie et al., 1998).

In the present work, to improve the utility of the AES, we have included coding elements in the optimized transfer vectors for the co-expression of GFP from *Aequorea victoria*. This marker of gene expression has the unique feature of being detectable in living cells and whole organisms. Its inclusion assists in the process of identifying recombinant AdV plaques and can be used to monitor gene transfer efficiency. Using GFP, we have recently developed a rapid and accurate titration method based on the measurement of gene transfer units by cytofluorometry (Couture et al., manuscript in preparation). The usefulness of this marker to monitor AdV-mediated gene transfer *in vivo* was illustrated by the co-detection of GFP and dystrophin in fixed muscles by histo-immunostaining as well as the development of a protocol to quantify GFP expression in muscle cells extract with a fluorometer (Couture et al., manuscript in preparation). Expression of GFP can occur independently of a gene of interest or can be linked through expression of a dicistronic message in which GFP is encoded as the second cistron (Mosser et al., 1997a). Using mutants of GFP with altered fluorescence properties (Palm et al., 1997; Stauber et al., 1998) increases the sensitivity of detection such that GFP expression can be easily detected in non-replicating cells that have been infected by a single AdV. Transfer vectors with expression cassettes for either GFP or the blue fluorescent protein (BFP) allow the independent identification of cells expressing more than one transgene.

Materials and methods

Cell culture and virus infection

HeLa cells expressing rtTA (HeLa-rtTA) and A549 cells expressing tTA (A549-tTA) have been described previously (Massie et al., 1998). A KB cell line, obtained from the American Type Culture Collection, expressing rtTA was generated by the same method described for the HeLa-rtTA cell line. Cell culture and virus production was performed as described (Garnier et al., 1994; Massie et al., 1995). Recombinant viruses were generated by *in vivo* homologous recombination between overlapping sequences of linearized transfer vectors and Ad5/ΔE1ΔE3 genomic DNA as detailed in Jani et al., (1997), with the following modifications. After the positive identification of Ad recombinants, they were plaque purified and amplified on BMAdE1 cells, an A549 cell line expressing AdE1 proteins from a vector designed to eliminate the generation of replication competent Ad (RCA) (Massie, 1998). BMAdE1 clone 78 was used for plaque purification for one or two rounds depending on the purity of the AdV clones. Subsequently, selected viral clones were amplified on BMAdE1 clone 220 up to 3×10^7 cells (passage # 3). Since the yield on BMAdE1 is about 5-fold lower than in 293 cells, AdV stocks were then further amplified in suspension culture of 293 cells (up to 3 to 6×10^9 cells) for only 2 passages, thereby minimizing the occurrence of RCA. The absorption conditions for infection and titration, either by plaque assay or by the gene transfer units method (Couture et al., manuscript in preparation), were done following the protocol of Mittereder et al., (1996) in minimal volumes (ex., 1.5 mL in 60 mm plates) for 6 to 16 hr in order to insure optimal entry of the virus. In our experience, this adsorption method can increase the uptake of Ad by a factor of 5 to 10 as compared to the previously described infection conditions of 2 hr incubation in a larger volume (ex., 3 mL in 60 mm plates). Thus, using the gene transfer units method, more than one third of Ad physical particles can be shown to be infectious (Couture et al., manuscript in preparation).

Transfer vectors and recombinant viruses

The transfer vector pAdTR5, which contains a modified tetracycline-regulated expression cassette, and pAdCMV5, which has an optimized human CMV-IE promoter expression cassette, have been described (Massie et al., 1998). Recombinant adenoviruses with

tetracycline-regulated expression of the herpes simplex virus ribonucleotide reductase large subunit protein R1 (AdTR5-R1), an N-terminal deleted R1 protein (AdTR5-ΔR1) were described previously (Massie et al., 1998).

AdV's expressing either the adenovirus E1B-19K protein or the human methenyl-tetrahydrofolate synthetase (MTHFS) protein was generated using the pAdTR5 transfer vector into which was cloned, in the BglII site, a PCR fragment containing either the E1B-19K coding region from pXC38 (McKinnon et al., 1982) or the MTHFS cDNA (Dayan et al., 1995) with BamHI ends and an optimized Kozak consensus sequence flanking the ATG initiation codon. All recombinant DNA molecules were constructed by standard cloning procedures. Restriction enzymes, modification enzymes and Vent polymerase were purchased from New England Biolabs (Beverly, Mass.)

The transfer vector pAdTR5-DC/GFP was constructed from pAdTR5 through a number of intermediate modification and cloning steps. First, a unique FseI site was added at the position corresponding to map unit 0 of the Ad5 genomic sequence. This creates a convenient 8 base pairs cleavage site for plasmid linearization prior to transfection. This was accomplished by digestion of pAdTR5 with EcoRI and BglII followed by ligation with a PCR fragment derived using the pAdTR5 plasmid as a template and primers that retained EcoRI and BglII ends but included an FseI site downstream from the EcoRI site to generate the plasmid pAdTR5F. The BglII site in this plasmid, which is located close to the Ad5 ITR, was next destroyed by digestion with BglII followed by blunt ending with Klenow and re-ligation. Subsequently a unique BglII site was added 5′ of the TR5 promoter. For this the plasmid was cleaved with BamHI and XhoI and the fragment that was removed was replaced with a PCR fragment derived using pAdTR5 as a template and primers that retained the XhoI site at the 5′ end but generated a 3′ end with BamHI, PmeI and BglII sites. This plasmid, pAdTR5FMCS, was used to finally generate pAdTR5-DC/GFP and the K7 series of plasmids described below. A plasmid, pTR5-DC/GFP, was used as the source of a fragment containing the encephalomyocarditis virus internal ribosome entry site (IRES) and GFP. This plasmid was derived from pTR-DC/GFP (Mosser et al., 1997a) by the insertion of a PCR fragment containing the adenovirus tripartite leader sequence using pAdBM5 (Massie et al., 1995) as a template and primers that added SacII ends such that the 3′ end also contains BglII site upstream from the SacII site. The pTR5-DC/GFP plasmid was digested with BglII and partially digested with BamHI to remove the IRES-GFP encoding containing fragment. This fragment was cloned into BamHI-BglII digested pAdTR5FMCS to generate pAdTR5-DC/GFP (Figure 1A). A described earlier (Mosser et al., 1997a) the GFP in pTR-DC/GFP is the S65T mutant (Heim et al., 1995).

pAdTR5-DC/HSP70-GFP was constructed by combining sequences from the plasmids pAdTR5F and pTR5-DC/HSP70-GFP (Mosser et al., 1997b). First, the unique EcoRI site in pAdTR5F was destroyed by digestion with EcoRI and blunt ending with Klenow followed by ligation. An EcoRI fragment from pTR5-DC/HSP70-GFP, containing the IRES sequence and including a BamHI site was excised and retained. The remainder of the plasmid was re-ligated and then a BamHI fragment encoding hsp70 and GFP was removed and cloned into the unique BamHI site in pAdTR5F. The resulting plasmid was then digested with EcoRI and the EcoRI fragment encoding the IRES was returned in its proper orientation to produce pAdTR5-DC/HSP70-GFP.

All of the K7 series of plasmids (Figure 1B) were derived from pAdTR5FMCS. Initially, this plasmid was digested with BglII and BamHI and a fragment containing the CMV-IE promoter and either the red-shifted GFP gene (GFPrg25) or the BFP gene (BFPrg50) from the plasmids QBI25 and QBI50 (Quantum Biotechnologies Inc., Montréal, Canada) were inserted (we refer to GFPrg25 and BFPrg50 as GFP_Q and BFP_Q respectively). These fragments have 5′ BglII and 3′ BamHI ends. To add cloning sites for insertion of a gene of interest and a polyA sequence downstream from the TR5 promoter a PCR fragment was generated containing the β-globin polyA sequence with 5′ BglII and PmeI sites and a 3′ BamHI site using the plasmid pAdCMV5 as a template. After digestion with BglII and BamHI the PCR product was cloned into the BglII cut GFP_Q and BFP_Q Ad plasmids to generate pAdTR5-K7/GFP_Q and pAdTR5-K7/BFP_Q (Figure 1B). The K7 plasmids with the constitutive CMV5 and MLP5 promoters were created by replacing the TR5 promoter in the pAdTR5-K7/GFP_Q and pAdTR5-K7/BFP_Q, after removal by digestion with AflII and XhoI, with a 1134 bp AflII/XhoI fragment containing the CMV5 promoter from pAdCMV5 or a 811 bp AflII/XhoI fragment containing the MLP promoter from pAdBM5 (Massie et al., 1995). This resulted in the generation of the transfer vectors pAdCMV5-K7/GFP_Q, pAdCMV5-K7/BFP_Q,

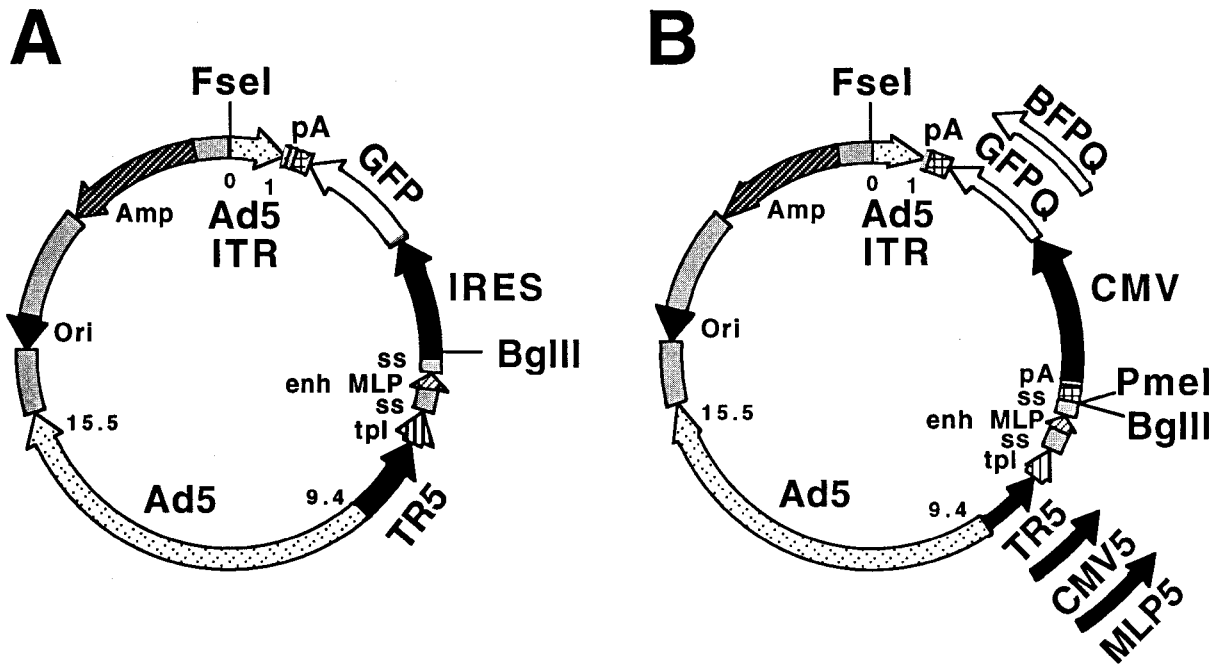

Figure 1. Schematic representations of the Ad transfer vectors encoding GFP. The dicistronic GFP expression cassette (A) and the independent GFP expression cassette (B) were derived from pAdTR5 and pAdCMV5 (Massie et al., 1998) and from pAdBM5 (Massie et al., 1995). The dicistronic cassette encodes the S65T mutant of GFP placed downstream of the encephalomyocarditis virus internal ribosome entry site (IRES). The independent cassettes encode either a red shifted mutant of GFP, GFP_Q, or a blue fluorescent protein, BFP_Q. Expression of an inserted gene of interest (into either the BglII in A or either the BglII or PmeI sites in B) is under the control of either a tetracycline-regulated promoter (TR) or the constitutive promoters CMV5 and MLP5. Each of the promoters is followed by the Ad5 tripartite leader sequence (tpl) and the MLP enhancer sequence (enh MLP) flanked by splice donor and acceptor sequences (ss). The Ad5 (Ad5 genome map units 9.4–15.5) and Ad5 ITR (m.u. 0–1) regions are Ad5 subgenomic sequences involved in homologous recombination used to generate Ad recombinants. A unique 8 base pairs FseI site can be used to linearize the plasmid prior to transfection.

pAdMLP5-K7/GFP_Q, and pAdMLP5-K7/BFP_Q (Figure 1B).

The AdCMV5-GFP virus, which expresses the S65T mutant of GFP under the control of the optimized CMV-IE promoter will be described elsewhere (Couture et al., in preparation). The plasmid pAdCMV5-GFP_Q was prepared by cloning a PCR product with BamHI ends encoding the red-shifted mutant of GFP (GFP_Q) from the plasmid pQBI25 (Quantum Biotechnologies Inc., Montréal, Canada) into BamHI cut pAdCMV5 (Massie et al., 1998) and was used to generate the virus AdCMV5-GFP_Q. The virus AdCMV-GFP_Q was generated using the transfer vector pAdMLP5-K7/GFP_Q. An adenovirus expressing β-galactosidase, AdCMV-LacZ (Ascadi et al., 1994), was used as a control in experiments with cells infected with adenoviruses expressing GFP.

Measurements of protein expression

For analysis of recombinant protein synthesis, 60 mm petri dishes of subconfluent cells were infected at a density of $0.5–1 \times 10^6$ cells/dish. At different times post-infection (p.i.), total protein extracts were prepared by lysing PBS washed cells with 2% SDS in 80 mM Tris-HCl pH 6.8 and 10% glycerol. Protein analysis by SDS-PAGE and Western blotting was performed as described previously (Lamarche et al., 1990). GFP levels were quantitated by western blotting using an anti-GFP monoclonal antibody (11EB, Quantum Biotechnologies Inc., Montréal, Canada) followed by incubation with a horseradish peroxidase conjugated goat anti-mouse antibody (Amersham Corp., Arlington Heights, Ill.) and ECL detection reagents (Amersham).

GFP fluorescence was measured by flow cytometry. Exponentially growing cells (1×10^6), seeded in duplicate 60 mm plates, were infected with AdCMV5-GFP, AdCMV5-GFP_Q or AdCMV-GFP_Q at various

MOIs. After 48 hr at 37 °C the cells were fixed with 2% paraformaldehyde for 30 min at 4 °C. GFP emission was analyzed using an EPICS XL-MCL flow cytofluorometer (Coulter, Miami, USA) equipped with a 15-mW argon-ion laser and the following filters: 488-nm laser-blocking, 488-nm long-pass dichroic, 550-nm long-pass dichroic and 525-nm band-pass. The fluorescence index was calculated as the product of the percentage of GFP positive cells by the mean fluorescence value.

Results and discussion

Features of the adenovirus transfer vectors with positive selection

Previously we described two new adenovirus expression cassettes that allow for either constitutive or inducible expression of a gene of interest in non-permissive cells (Massie et al., 1998). The transfer vector pAdTR5 utilizes a tetracycline-regulatable promoter (Gossen and Bujard, 1992) while the pAdCMV5 vector contains an optimized CMV-IE promoter/enhancer. To increase the utility of these transfer vectors we have included elements for the co-expression of GFP as described in Material and Methods (Figure 1). In the plasmid pAdTR5-DC/GFP (Figure 1A) the GFP gene, containing the S65T mutation (Heim et al., 1995), is encoded as the second cistron in a dicistronic expression cassette. A gene of interest can be cloned into the position of the first cistron by insertion into the unique BglII site. The two cistrons are separated by the encephalomyocarditis virus internal ribosomal entry site (IRES). We have previously shown that this dicistronic cassette can be used to facilitate the screening and selection of transfected cell lines stably expressing inducible gene products (Mosser et al., 1997a, b). Another series of transfer vectors that allow for the independent expression of GFP and a gene of interest were also assembled by including a GFP expression cassette (K7-GFP_Q) into either the pAdTR5, pAdCMV5 or pAdBM5 plasmids (Figure 1B). In these plasmids the expression of a red-shifted variant of GFP (GFP_Q), or a blue fluorescent protein (BFP_Q), is placed under the control of the standard CMV-IE promoter. Two unique cloning sites, BglII and PmeI, are available for insertion of a gene of interest. Three pairs of plasmids offer either tetracycline-inducible expression (pAdTR5-K7-GFP_Q/BFP_Q) or constitutive expression (pAdCMV5-K7-GFP_Q/BFP_Q, pAdMLP5-K7-GFP_Q/BFP_Q). All of the above transfer vectors can be used for (i) transient expression assays, (ii) to establish stable cell lines and (iii) to generate recombinant Ad's. In combination they offer the ability to express two different proteins with either constitutive or inducible expression and can be detected separately with co-expression of the blue or green fluorescent proteins.

Efficient protein production in absence of Ad replication

Although equally high levels of expression can be obtained with either the constitutive (AdCMV5) or the tetracycline-regulated (pAdTR5) AdV's, only the inducible system allows for the expression of potentially toxic proteins. This is because the generation of a recombinant Ad is accomplished *in vivo* in permissive cells transfected with the transfer vector and E1-deleted viral DNA. Constitutive expression of proteins, encoded by the transfer vector, that can interfere with viral replication or assembly will prevent the successful generation of a recombinant virus. This was assumed to be the reason for our previous unsuccessful attempts to generate an Ad expressing N-terminal deletions of the herpes virus ribonucleotide reductase protein R1 (ΔR1). However, a recombinant Ad with tetracycline-regulated expression of ΔR1 was readily obtained (Massie et al., 1998). Figure 2 shows examples of high level regulated expression of a number of proteins using AdTR5 vectors in KB cells that stably express rtTA. Levels of expression range from 30% TCP for AdTR5-R1 to 10–15% TCP for the others. Expression is undetectable in cells infected in the absence of the inducer, doxycycline (compare lanes 2 and 3). As we have shown previously (Massie et al., 1998), high level co-expression of two proteins can be easily achieved by co-infection of cells with each of the AdV. This is shown in lane 4 where cells were infected with an equal MOI (400 pfu) of the AdTR5-ΔR1 and AdTR5-DC/hsp70-GFP viruses. Expression levels obtained after infection with either of these viruses singly is shown in lanes 5 and 6. Two other examples of proteins that were expressed at high levels using the tetracycline-inducible AdV are the human enzyme MTHFS (lane 7) and the Ad E1B-19K protein (lane 8). Another AdV expressing the MTHFS under the control of the CMV5 promoter was also generated and it produced the enzyme at the same level as AdTR5-MTHFS (data not shown) like this was the case for R1 expressed in both vectors (Massie et al., 1998).

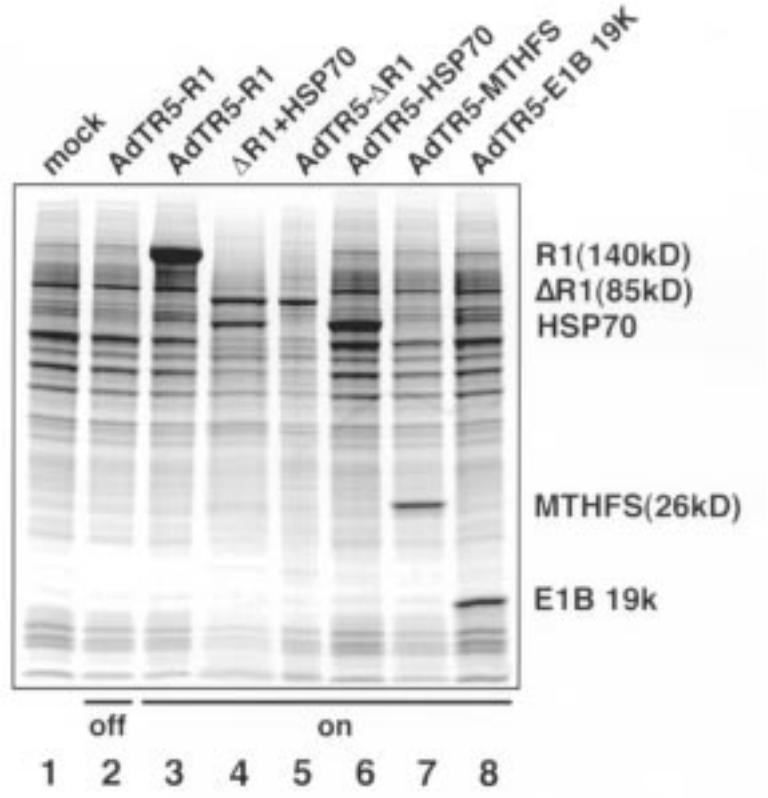

Figure 2. High-level protein production with AdTR5 vectors in KB-rtTA cells. Shown is a Coomassie blue stained SDS-PAGE gel of protein extracts (10 μg/lane) from cells infected at an MOI of 400 and collected 40 hr. later. Cells were infected with recombinant Ad's encoding the herpes simplex virus ribonucleotide reductase large subunit R1 (lanes 2 and 3), an R1 N-terminal deletion ΔR1 (lanes 3 and 4), the human 70 kDa heat-inducible protein hsp70 (lanes 4 and 6), the human methenyltetrahydrofolate synthetase enzyme MTHFS (lane 7) and the adenovirus E1B-19 K protein (lane 8). The rtTA inducer, doxycycline (1 μg mL^{-1}) was added to the culture medium at the time of infection. Lane 2 shows the absence of R1 protein synthesis in cells infected with the AdTR5-R1 virus without addition of doxycycline. An extract from non-infected cells (mock) is shown in lane 1.

The expression of R1 in KB-rtTA cells at 30% TCP is slightly higher than what was obtained in A549-tTA or HeLa-rtTA with AdTR5-R1 but is equivalent to the expression level obtained in the aforementioned cell lines following co-infection with an AdV encoding tTA (Massie et al., 1998). This suggest that this particular clone of KB cells is expressing rtTA at a level higher than tTA or rtTA are expressed in A549 or HeLa cells respectively, and also that its expression level rivals the one obtained following AdVCMVtTA co-infection. Taken together our results show that the AES is a very efficient protein production system allowing to express both toxic and non-toxic proteins at near saturation levels in absence of viral replication in a wide variety of mammalian cells. The absence of AdV replication is a feature that facilitates protein purification since no other abundant viral proteins are produced. Moreover, owing to the absence of viral replication and ensuing host protein shut-off, the production host can be maintained in good physiological state for prolonged periods of time (weeks) and continue to synthesize the recombinant protein, that could be harvested periodically, if secreted in the medium. Indeed, we have shown by radiolabeling with [^{35}S]methionine that protein synthesis reaches a maximun at 24 hr pi and can be maintained for at least one week (Massie et al., 1998; and unpublished data).

Cytotoxicity following overexpression of E1B-19K

The E1B-19K protein plays an essential role in adenovirus replication by inhibiting the apoptotic effects that are associated with the expression of E1A (White et al., 1991). Constitutive expression of E1B-19K in transfected cell lines confers upon them an increased

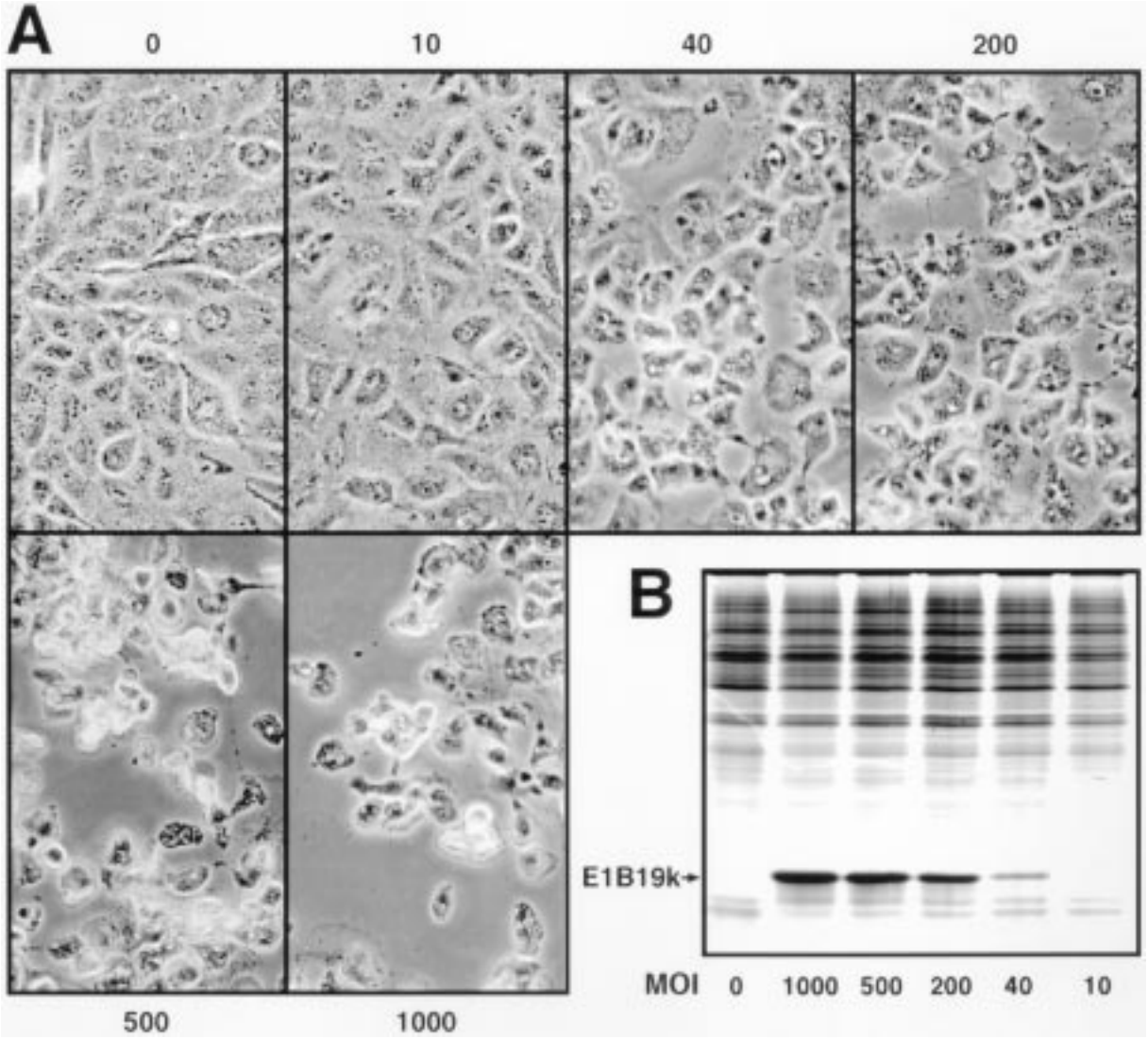

Figure 3. High level expression of the anti-apoptotic protein E1B-19K is lethal in A549-tTA cells. Morphological appearance and E1B-19K expression levels in cells infected with the AdTR5-E1B 19K AdV at MOI's of 10, 40, 200, 500 and 1000. Doxycycline (1 μg mL^{-1}) was added to each of the cultures, and to the non-infected cells (0), at the time of infection. Cells were photographed and then collected for SDS-PAGE 40 hr. after infection (15 μg of protein cell extract/lane). Cytotoxicity is first seen by 24 hr. in cells infected at an MOI of 40. Expression of E1B-19K represents approximately 2% of total cell protein in cells infected at this MOI.

resistance to apoptosis in response to a number of diverse stimuli (reviewed in Teodoro and Branton, 1997). Surprisingly, we were unable to generate a recombinant adenovirus expressing this protein under the control of the strong CMV5 promoter. Our ability to readily generate an E1B-19K expressing adenovirus with the tetracycline-inducible AdV led us to suspect that high level expression of E1B-19K was cytotoxic, unlike the anti-apoptotic function that it possesses when expressed at more moderate levels. This is clearly shown in Figure 3 where infection of A549-tTA cells at an MOI of 40 pfu or greater led to intense vacuolization and a loss of attached cells from the culture dish. Cytotoxicity was evident at expression levels that were approximately 2% of TCP whereas expression at lower levels resulted in protection against apoptosis induced by various treatments (Langelier et al.; manuscript in preparation). No cytotoxicity was observed when cells were infected in the presence of anhydrotetracycline which prevents

tetracycline-regulated expression in tTA-expressing cells (not shown). The cytotoxicity occurring as a result of high level E1B-19K protein expression is not simply due to a general effect related to such high levels of protein production since this level of protein expression was well tolerated for a number of other proteins (Figures 2 and 5). E1B-19K is targeted to membranes of the nucleus and endoplasmic reticulum, and is associated with lamins (Rao et al., 1997). Toxicity may be the result of mislocalization of the E1B-19K protein or inappropriate or unregulated associations with essential cellular proteins when expressed at high levels. This is another example of a cytotoxic protein expressed using the tetracycline-inducible system in AdV (Hu et al., 1997; Massie et al., 1998). We are also expressing the pro-apoptotic proteins Bad and Bax and preliminary results indicates that only the inducible vector can generate viable Ad recombinants.

Autofluorescent protein expression for positive selection of Ad recombinants

Co-expression of GFP in the AdTR5-DC based cassettes simplifies the process of recombinant virus identification and greatly increases the utility of the recombinant Ad. Generally, recombinant Ad's are identified as plaques on monolayer cultures of 293 cells that have been overlaid with agarose. Individual plaques are verified as being genuinely recombinant by screening methods that involve plaque elution and amplification followed by dot blotting, PCR, or SDS-PAGE and western blotting to verify the presence of the recombinant protein (Jani et al., 1997). By contrast, recombinant Ad's that co-express GFP can be easily identified by direct observation with an inverted fluoresecent microscope. Since GFP expression is detectable in a large percentage of cells (typically 10 to 50%) one day after the transfection, it can be used to monitor the efficiency of transfection, a crucial parameter for the successful generation of recombinant Ad's. By the time the first plaques are visible, 7 to 10 days post-transfection, the percentage and intensity of fluorescent cells has diminished substantially due to the transient nature of GFP expression. Although, pockets of green cells are still present and can be confused with real plaques, a careful morphological examination allows to readily discriminate between them. Indeed, the cells within a plaque display a typical cytopathic effect and are more fluorescent. Plaques that are green can be rapidly identified and purified by subsequent rounds of infection and selection of individual green plaques, allowing to save as much as 3 to 4 weeks on the selection and plaque purification process.

Expression from the TR5 promoter in the 293 cell line is sufficient to identify green plaques in the uninduced state, although the level of GFP fluorescence is about 25-fold less than when induced. This is due to the leakiness of the TR5 promoter in 293 cells, likely resulting from the transactivation of the minimal CMV TATA box by the AdE1A proteins in combination with the high copy number of the vectors following transfection or Ad replication as discussed in our previous report (Massie et al., 1998). Obviously, this intrinsic leakiness might preclude the generation of AdV's expressing very toxic proteins, and so far we have found at least one gene that we were unable to rescue in AdV. Work is in progress to minimize the leakiness of the uninduced state while preserving the strong activity of the fully induced promoter. Despite this limitation, we have successfully produced recombinant Ad's with the AdTR5-DC/GFP transfer vector expressing the chaperone hsp70; the cytokines EPO, IL-2 and TGFβ; the G-coupled receptor protein hDOR; the adenovirus protein E1B-19K; and the pro-apoptotic proteins Bad and Bax using this strategy. By comparison to a vector in which the GFP gene is next to the TR5 promoter, the expression of GFP in the second cistron is reduced by 2- to 4-fold depending on the first gene in the dicistronic construct (not shown). The reason for this reduced expression is currently under investigation, but it is not a major problem since GFP is readily detectable at that level. However, since bright green fluorescence can only be seen when the dicistronic message is induced to high levels, and since this would prevent the generation of recombinant Ad's expressing potentially toxic proteins, we have also constructed transfer vectors in which the expression of GFP is independent of the gene of interest (pAdTR5-K7/GFP$_Q$). Recombinant Ad's with tetracycline-regulated expression of HSV ribonucleotide reductase subunit R1, the p53-regulated protein p21, the G-coupled receptor protein hDOR, the adenovirus proteins E1A, E1B-19K, E3-gp19K and E3-14.7K, as well as the murine cytokines IL-6 and IL-10 have been generated with the pAdTR5-K7/GFP$_Q$ transfer vector. Preliminary characterization of some of these recombinant Ad's indicated that not only the GFP reporter, but also the other gene in the construct was expressed at higher levels in the independent double cassettes as compared to the di-

cistronic vectors (not shown). Interestingly, so far every GFP positive plaque tested also produced the other transgenes showing that selection of AdV with this procedure is reliable. Given the structure of the tandem expression cassettes in the vector, it is formally possible that rearrangements of the AdV could occur through recombination between homologous sequences in the transfer vector generating unstable AdV stocks. However, this has not been observed with the limited number of Ad recombinants we have analyzed at this point.

An important consideration in the use of GFP for the identification of recombinant Ad's is the level of GFP expression that is minimally required for detection. Since high sensitivity would be a beneficial attribute, we compared the expression level and fluorescence intensity of GFP in 293 cells infected with AdV's expressing either the S65T mutant of GFP (Heim et al., 1995) or a red-shifted variant GFP_Q (Palm et al., 1997; Stauber et al., 1998). Figure 4 shows that while expression levels of both GFP S65T and GFP_Q are similar when expressed from the AdCMV5 viruses (Figure 4A and B, lanes 2 and 3) the fluorescence index of the GFP_Q is almost 12-fold higher than that of GFP S65T (93 vs. 8). When expressed under the control of the weaker promoter in the AdCMV virus, GFP_Q expression is approximately 6-fold less than that obtained with AdCMV5/GFP_Q (compare Figure 4B lane 5 which shows a 1:9 dilution of the extract from AdCMV5-GFP_Q infected cells with lane 6 which is the undiluted extract from AdCMV-GFP_Q infected cells). However, the fluorescence index is still about 2-fold higher than that of GFP S65T expressed under the control of the strong CMV5 promoter (17 vs. 8). Therefore, using a GFP variant that has been optimized for GFP fluorescence makes it possible to detect GFP positive cells without the need to express high amounts of the reporter protein.

Functional studies in non-permissive cells with autofluorescent proteins

We performed similar experiments in cells that do not support virus replication to determine whether GFP fluorescence could be detected in cells that received a single copy of the gene and also whether the level of GFP fluorescence could be directly correlated with the copy number delivered. This is an important consideration in applying AdV's for functional studies *in vitro* or *in vivo* gene therapy. Figure 5A shows GFP_Q protein levels in HeLa cells infected with AdCMV5GFP_Q at MOI's of 100, 1000 and 5000 and is compared to levels of GFP_Q in HeLa cells infected with AdCMV-GFP_Q at an MOI of 5000 and 293 cells infected with AdCMV5-GFP_Q at an MOI of 10. Equivalent amounts of GFP_Q can be produced in non-replicating cells at a high MOI (5000) as compared to 293 cells where the virus replicates to a high copy number (compare lanes 2 and 6). GFP_Q is undetectable by Coomassie staining in HeLa cells infected with AdCMV5-GFP_Q at an MOI of 100 (lane 4) or with AdCMV-GFP_Q at an MOI of 5000 (lane 7). Western blot analysis (Figure 5B) shows that the amount of GFP_Q produced by the standard CMV-IE promoter (lane 7) was similar to that produced using a 50-fold lower MOI of the stronger CMV5 promoter (lane 4). Increasing the MOI in both AdCMV5-GFP_Q and AdCMV-GFP_Q infected cells gives a proportional increase in the amount of GFP fluorescence (Figure 5C). The fluorescence index of GFP_Q in AdCMV5-GFP_Q infected cells is far superior to that of AdCMV-GFP infected cells (approximately 12-fold higher over an MOI range of 0.25 to 10). This is somewhat higher than the difference measured in 293 cells where the fluorescence index of AdCMV5-GFP_Q infected cells was about 6-fold higher than in AdCMV-GFP_Q infected cells (Figure 4B, 93 vs. 17). Although the fluorescence intensity is weaker in HeLa cells infected with the AdCMV-GFP_Q virus it is sufficient to detect GFP_Q in cells containing a single copy of the GFP_Q gene. Infection with an MOI of 1, as normalized in 293 cells (where an MOI of 1 gives 50–60% positive cells), resulted in the detection of 28% GFP positive cells with the AdCMV5-GFP_Q virus and 16% positive cells with the AdCMV-GFP_Q virus. The sensitivity is therefore about 2-fold less for AdCMV5-GFP_Q and 4-fold less for AdCMV-GFP_Q at an MOI of 1 in HeLa cells relative to 293 cells where the virus replicates and reaches saturation levels for GFP.

A proportional increase in the fluorescence index relative to MOI can be measured up to an MOI of 1000 in AdCMV5-GFP_Q infected HeLa cells (Figure 5D). For example, increasing the MOI from 10 to 100 or 1000 gives a 10- and 100-fold increase in fluorescence index. However, increasing the MOI from 1000 to 5000 or 10 000 does not give a proportional 5- and 10-fold increase but rather less than 2-fold going from 1000 to 5000. This may be because the uptake of the virus is near saturation at such high MOI's, or because GFP_Q protein synthesis levels are nearing saturation, or because of alteration of cell physiology at such high MOIs. The fact that in complementing

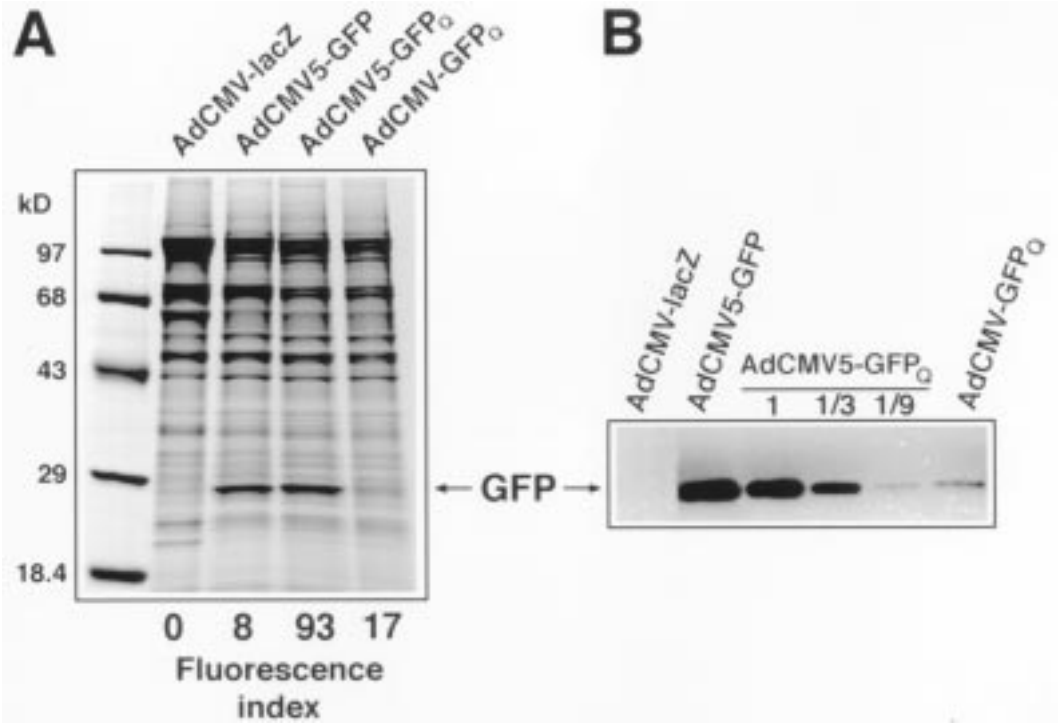

Figure 4. Comparison of GFP expression and fluorescence levels in 293 cells infected with AdV's expressing either the S65T mutant of GFP (GFP) or the red-shifted variant (GFP_Q) under the control of either the CMV-IE or CMV5 promoter. Coomassie blue stained gel (A) and western blot analysis (B) of GFP expression levels in protein extracts from 293 cells (10 μg/lane) infected with an MOI of 10 and collected 24 hr. after infection. The level of GFP fluorescence in the infected cells, as measured by flow cytometry, is indicated below the gel shown in 'A'. Dilutions (1/3 and 1/9) of the extract from the cells infected with the AdCMV5-GFP_Q virus are shown in panel 'B' in order to estimate the level of GFP expression in these cell relative to that of cells infected with the AdCMV-GFP_Q virus. Cells infected with a CMV-based promoter expressing lacZ (AdCMV-lacZ) is included as a control.

293 cells, in which the copy number of the vector can be as high as 100 000, a difference of 6-fold in favor of CMV5 as compared to CMV-IE was measured whereas in HeLa cells at lower copy number a 12-fold difference is obtained, suggest that at copy numbers greater than 5000 a saturation of GFP expression is occurring. The results of the western blot analysis, in agreement with the fluorescence index measurements, shows that although an approximate 10-fold increase in GFP_Q levels was achieved by increasing the MOI from 100 to 1000, an increase from 1000 to 5000 resulted in only less than a 2-fold increase in GFP_Q levels (Figure 5B). Thus, for functional studies gene expression can be increased proportionally up to an MOI of 1000 reaching near saturation, although more typically, studies should be conducted at lower MOIs in order to minimize any side effects associated with the viral load. Owing to the increased sensitivity of GFP_Q, we were able to established the minimal MOI (around 3 to 5) required to transfer a gene in almost 100% of HeLa cells with AdV.

Conclusions

Addition of elements for the co-expression of GFP significantly increases the utility of the AES that we have previously shown is capable of producing high levels of recombinant proteins. Use of a tetracycline-inducible expression system makes it possible to generate recombinant Ad's expressing proteins that are toxic and capable of interfering with Ad replication. Co-expression of GFP assists in the identification of recombinant Ad's since the positive plaques can be easily seen with an inverted fluorescent microscope. Furthermore, co-expression of GFP makes it possible to gauge the level of transgene expression following infection. For *in vivo* applications it allows for the identification of productively infected cells and so can be used to measure the effectiveness of the gene transfer protocol.

Expression of GFP from an independent promoter (K7 vectors) gives high levels of GFP fluorescence without expression of the gene of interest (pTR5 vec-

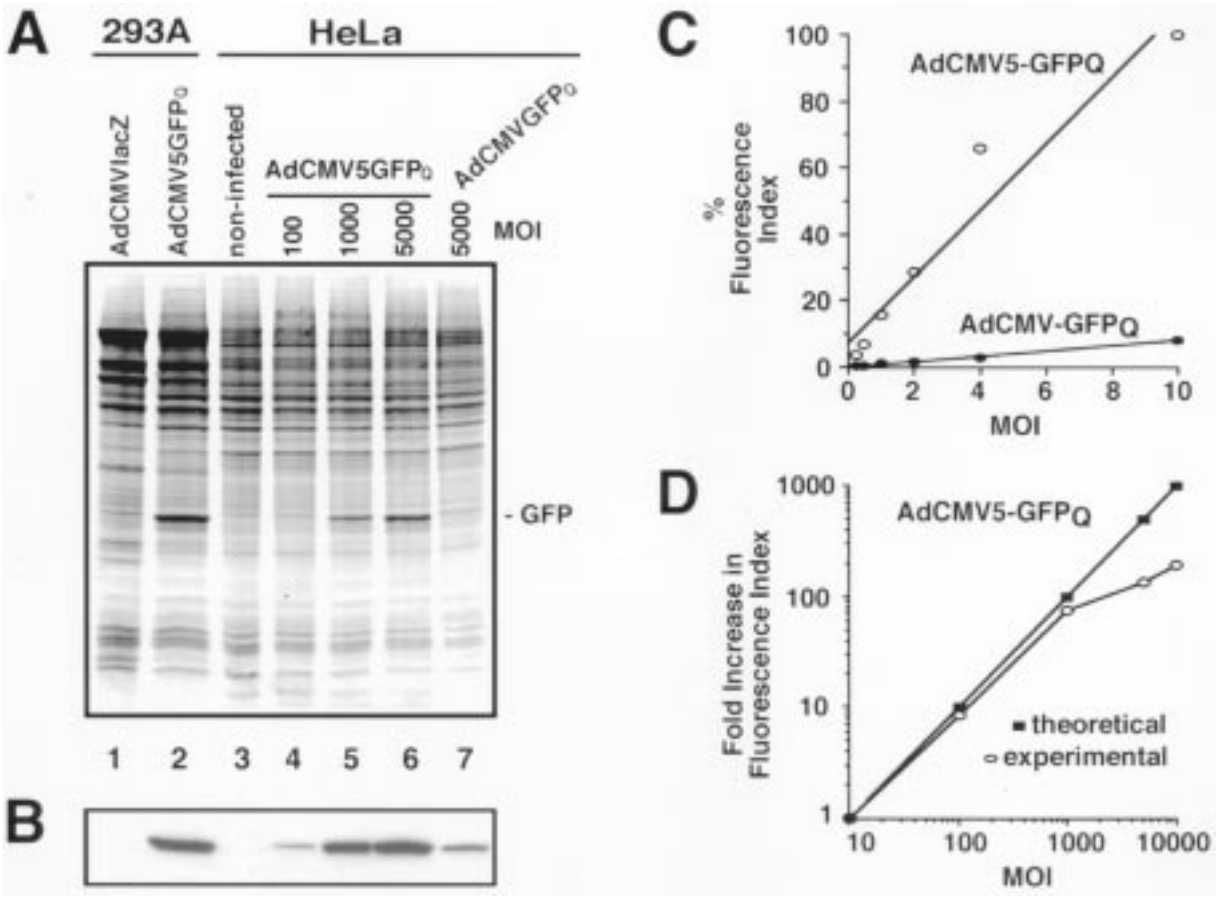

Figure 5. Examination of GFP expression and fluorescence levels in HeLa-rtTA cells infected with AdV's expressing GFP_Q under the control of either the CMV-IE or CMV5 promoter. Coomassie blue stained gel (A) and western blot analysis (B) of GFP expression levels in protein extracts (10 μg/lane) from 293 cells (lanes 1 and 2) and HeLa-rtTA cells (lanes 3–7) infected with AdCMV5-GFP_Q (lanes 2, 4, 5, 6, and 7) at the indicated MOI. Also shown are 293 cells infected with AdCMV-lacZ (lane 1) and non-infected HeLa-rtTA cells (lane 3). Cells were collected 48 hr. after infection. (C) Fluorescence index of HeLa-rtTA cells infected with AdCMV5-GFP_Q or AdCMV-GFP_Q at MOI's ranging from 0.25 to 10. The fluorescence index is plotted as a percentage of the maximum value obtained with cells infected with AdCMV5-GFP_Q at an MOI of 10. GFP fluorescence in paraformaldehyde fixed cells was measured 48 hr. after infection. (D) Correlation between the fluorescence index and MOI for HeLa-rtTA cells infected with AdCMV5-GFP_Q. The fold increase in fluorescence index is plotted relative to the value obtained with cells infected at an MOI of 10. The theoretical value, assuming a log increase in fluorescence index for each log increase in MOI, is plotted (square symbol) for comparison to the experimentally measured value (open circle).

tors) and the recombinant plaques are much easier to identify than those generated with the dicistronic GFP vector. GFP can be seen in plaques on 293 cells generated from the pTR5-DC/GFP vector due to the low level of expression from this promoter in these cells in the uninduced state. These plaques can be rapidly verified by infection of 293-tTA cells (Massie et al., 1998) with the eluted viruses. Since the red-shifted variant of GFP present in the K7 transfer vectors is much brighter than the S65T mutant GFP, we have recently constructed dicistronic transfer vectors with both GFP_Q and BFP_Q. An advantage of the dicistronic configuration is that the levels of fluorescence are directly proportional to the level of expression of the gene of interest. When using the tetracycline-inducible system, dicistronic expression of GFP identifies which cells are expressing a gene of interest following induction and the level of expression can be inferred from the level of GFP fluorescence. Also, dicistronic expression of GFP will give high levels of GFP expression in any cell that co-expresses the transactivator, while the K7 vectors may be limited to cells in which the CMV-IE promoter is strongly activated which is the case for the majority of the cells with a few exceptions (Hu et al., 1997; Xu et al., 1995).

Acknowledgements

We thank Arik Elfassy for measurement of GFP expression in AdV with the dicistronic expression cassette and Lucie Bourget for technical assistance with the cytofluorometer. This work was supported by grants from the National Research Council and the Medical Research Council of Canada. This is a NRC publication No. 41438.

References

Acsadi G, Jani A, Massie B, Simoneau M, Holland P, Blaschuk K and Karpati G (1994) A differential efficiency of adenovirus-mediated *in vivo* gene transfer into skeletal muscle cells of different maturity. Hum Mol Genetic 3: 579–584.

Acsadi G, Massie B and Jani A (1995) Adenovirus-mediated gene transfer into striated muscles. J Mol Med 73: 165–180.

Berkner KL (1988) Development of adenovirus vector for the expression of heterologous genes. BioTechniques 6: 616–629.

Berkner KL (1992) Expression of heterologous sequences in adenoviral vectors. Curr Top Microbiol Immunol 158: 39–66.

Dayan A, Bertrand R, Beauchemin M, Chahla D, Mamo A, Filion M, Skup D, Massie B and Jolivet J (1995) Cloning and characterization of the human 5,10-methenyltetrahydrofolate synthetase-encoding cDNA. Gene 165: 307–311.

Garnier A, Côté J, Nadeau I, Kamen A and Massie B (1994) Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells. Cytotechnology 15: 145–155.

Gossen M and Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89: 5547–5551.

Gossen M, Freundlieb S, Bender G, Müller G, Hillen W and Bujard H (1995) Transcriptional activation by tetracyclines in mammalian cells. Science 268: 1766–1769.

Graham FL and Prevec L (1992) Adenovirus-based expression vectors and recombinant vaccines, In RW Ellis (Ed.) Vaccines: New Approaches to Immunological Problems. Butterworth-Heinemann, Boston, Mass, pp. 363–390.

Heim R, Cubitt AB and Tsien RY (1995) Improved green fluorescence. Nature 373: 663–664.

Hu SX, Ji W, Zhou Y, Logothetis C and Xu HJ (1997) Development of an adenovirus vector with tetracycline-regulatable human tumor necrosis factor alpha gene expression. Cancer Res 57: 3339–3343.

Jani A, Lochmüller H, Acsadi G, Simoneau M, Huard J, Garnier A, Karpati G and Massie B (1997) Generation, validation and large scale production of adenoviral recombinants with large inserts such as 6.3 kb human dystrophin cDNA. J Virolog Meth 64: 111–124.

Lamarche N, Massie B, Richer M, Paradis H and Langelier Y (1990) High level expression in 293 cells of the herpes simplex virus type 2 ribonucleotide reductase subunit 2 using an adenovirus vector. J Gen Virol 71: 1785–1792.

Massie B (1998) BMAdE1, a new complementing cell line for the large scale production of E1-deleted Adenovirus (Ad) vectors devoid of RCA (replication competent Ad). (filed on 26/04/96; Issued 09/98).

Massie B, Couture F, Lamoureux L, Mosser DD, Guilbault C, Jolicoeur P, Belanger F and Langelier Y (1998) Inducible overexpression of a toxic protein by an adenovirus vector with a tetracycline-regulatable expression cassette. J Virol 72: 2289–2296.

Massie B, Dionne J, Lamarche N, Fleurent J and Langelier Y (1995) Improved adenovirus vector provides herpes simplex virus ribonucleotide reductase R1 and R2 subunits very efficiently. Bio/Technology 13: 602–608.

McKinnon RD, Bacchetti S and Graham FL (1982) Tn5 mutagenensis of the transforming genes of human adenovirus type 5. Gene 19: 33–42.

Mittereder N, March K and Trapnell BC (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J Virol 70: 7498–7509.

Mosser DD, Caron AW, Bourget L, Jolicoeur P and Massie B (1997a) Use of a dicistronic expression cassette encoding the green-fluorescent protein for the screening and selection of cells expressing inducible gene products. Biotechniques 22: 150–161.

Mosser DD, Caron AW, Bourget L, Denis-Larose C and Massie B (1997b) Role of the human heat shock protein hsp70 in protection against stress–induced apoptosis. Mol Cell Biol 17: 5317–5327.

Palm GJ, Zdanov A, Gaitanaris GA, Stauber R, Pavlakis GN and Wlodawer A (1997) The structural basis for spectral variations in green fluorescent protein. Nat Struct Biol 4: 361–365.

Rao L, Modha D and White E (1997) The E1B 19K protein associates with lamins *in vivo* and its proper localization is reuired for inhibition of apoptosis. Oncogene 15: 1587–1597.

Stauber RH, Ilorie K, Carney P, Hudson EA, Tarasova NI, Galtanaris GA and Pavlakis GN (1998) Development and applications of enhanced green fluorescent protein mutants. BioTechniques 24: 462–471.

Stratford-Perricaudet L D and Perricaudet M (1994) Gene therapy: the advent of adenovirus, In John A Wolff (Ed.) Gene Therapeutics: Methods and Applications of Direct Gene Transfer. Birkhauser Boston, Cambridge, Mass, pp. 344–362.

Teodoro JG and Branton PE (1997) Regulation of apoptosis by viral gene products. J Virol 71: 1739–1746.

Trapnell BC and Gorzilia M (1994) Gene therapy using adenoviral vectors. Curr Opinion in Biotech 5: 617–625.

White E, Cipriani R, Sabbatini P and Denton A (1991) Adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. Virol 65: 2968–2978.

Xu ZZ, Krougliak V, Prevec L, Graham FL and Both GW (1995) Investigation of promoter function and animal cells infected with human recombinant adenoviruses expressing rotavirus antigen VP7sc. J Gen Virol 76: 1971–1980.