

Helper-dependent adenoviral vectors as gene delivery vehicles

Review Article

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Summary

Adenoviral (Ad)-mediated *in vivo* gene transfer and expression is limited in part by cellular immune responses to viral-encoded proteins. In an attempt to diminish these responses, we have previously developed and described helper-dependent (HD) Ad vectors in which the viral protein coding sequences are completely deleted. These vectors provided efficient delivery, and greater safety which represents a significant advance over existing Ad vectors. In addition, the inherent enhanced insert capacity (up to ~ 37kb) allows for the insertion of large or multiple genes, including expression regulatory sequences. Several drug-regulated gene expression systems are now available for controlling target gene transcription through the use of small-molecule inducing compounds. While early experiments have demonstrated the utility of inducible systems in cell culture and transgenic mice, continued evaluation of such systems in viral gene therapy vectors should lead to discoveries and improvements which will make them amenable for use in a therapeutic context. The generation of a gene therapy approach that combines both safe and efficient vector delivery of one or multiple genes of interest and a small molecule-controlled gene expression system will provide a powerful tool for therapeutic intervention.

I. Introduction

Gene therapy is a rapidly evolving technology for therapeutic intervention which involves delivery and expression of nucleic acid in a target cell to complement a genetic defect or deliver a new protein. The diversity in the nature of the expressed product is wide and ranges from expressing cellular enzymes, cellular or circulating proteins, secreted hormones, cytokines or growth factors, to immunogens, ribozymes, or antisense oligonucleotides. The main components of a gene therapy agent are the vector or delivery vehicle and the expression cassette, which is composed of the gene(s) of interest and the promoter elements controlling expression. Recently, intense research has evolved and is ongoing to identify the most suitable vector(s) for gene delivery. Viral and non-viral vectors are continuously being modified, examined and compared for safety, persistence and efficacy in delivery and mediation of gene expression. Among the most extensively studied viral vectors are the retroviral, adenoviral and adeno-associated, and among the non-viral vectors are the DNA-lipid

complexes (cationic liposomes), DNA-polylysine conjugates or delivery of naked DNA (Morsy et al., 1993a). The adenoviral vectors in specific have seen a great deal of modifications over the years. These modifications ranged from rendering them replication deficient (E1 deleted vectors or 1st generation) to completely deleting all viral protein coding regions (helper-dependent). We will review some data comparing the efficacy of helper-dependent vectors to that of 1st generation in terms of safety, longevity of expression and efficiency of gene delivery *in vivo*.

The final section of this review will briefly summarize recent reports evaluating the potential for ligand-inducible control of gene expression in the context of gene therapy vectors. Use of drug-regulated systems may introduce several levels of control to current gene therapy vectors (Harvey and Caskey, 1998), as therapeutic gene expression will remain transcriptionally silent until activated by the addition of an inducing compound. Ideally, this can provide for temporal control in cases where constitutive gene expression may not be required or even preferred. In

addition, control of expression levels within their therapeutic range may be afforded by appropriate dosing of the inducer. In cases where expression of a drug-regulated transcriptional transactivator is driven by a tissue-specific promoter, some degree of spatial control over gene expression may also be achieved.

II. Adenoviral vectors

Over the past few years adenoviruses (Ad) have taken a forefront position as gene delivery vehicles as a result of their numerous advantageous features. Their popularity as recombinant vectors is largely due to the successful and safe immunization of millions of US military recruits with enteric coated Ad4 and Ad7 to prevent against acute respiratory disease (ARD) outbreaks (Gaydos and Gaydos, 1995). In addition, the Ad genome is well characterized, and easily manipulated. Recombinant Ad vectors have been generated, with different deletions in their genome to render the vector replication deficient and to allow for insertion of foreign DNA sequences. Current generations of Ad vectors have insert capacity ranging from 7-9 Kb, and are deleted in one or more combinations of the early genes. Recombinant viruses are stable and stocks can be concentrated to titers higher than 10^{12} plaque forming units / ml. The virus has a broad cellular host range, its uptake is not restricted to dividing cells, has natural tropism to liver, lung and intestine which is dictated by route of delivery (vascular, inhalation and oral, respectively) and persists as an episome in infected cells. It encodes for a cascade of polypeptides including the capsid structural proteins, the hexones, pentons (fiber and penton base). Recent studies have shown (Ad) vectors to be among the most efficient gene transfer vehicles for both *in vitro* and *in vivo* delivery. The general utilization, however, of current Ad vectors for many gene therapy applications is limited by the transient nature of transgene expression observed (Muzzin et al., 1996, Stratford-Perricaudet et al., 1990, Herz and Gerard, 1993, Morsy et al., 1993a, Morsy et al., 1993b, Morsy et al., 1996). Several factors have been shown to contribute to and modulate the duration of Ad-mediated gene expression and the underlying immunogenicity of these vectors. These factors include "leaky" viral protein expression and/or the immunogenicity of the transgene that is delivered (Yang, 1995, Gahery-Segard et al., 1997, Kaplan et al., 1997, Tripathy et al., 1996, Worgall et al., 1997). The development of Ad vectors that are deleted in all viral protein-coding sequences offers the prospect of a potentially safer, less immunogenic vector with an insert capacity of up to approximately 37 kb (Mitani et al., 1995, Kochanek et al., 1996, Clemens et al., 1996, Fisher et al., 1996, Kumar-Singh and Chamberlain, 1996, Hardy et al., 1997, Lieber et al., 1996, Parks et al., 1996, Schiedner et al., 1998, Haecker et al., 1996). This vector requires viral regulatory and structural proteins which, when supplied in *trans*, can support packaging and rescue, and is thus named

helper-dependent (HD) (Parks et al., 1996). It is noteworthy however, to emphasize that such modifications would modulate the toxicity and enhance the safety of the HD vehicle itself, yet may or may not have an effect on the impact or extent of transgene immunogenicity.

A modified Ad vector has been generated such that it is completely devoid of viral protein encoding sequences (Morsy et al., 1998a). The new vector contains the ITR and packaging sequences, with an insert capacity of up to 37 kb. The propagation of this vector requires supplementation of the viral proteins in *trans*, which presently are supplied by co-propagation of a helper virus. Both viruses can be separated on a cesium gradient. Further more the helper virus is crippled by flanking the packaging signal sequence with lox sites that allow the excision of the intervening DNA, in the presence of *cre*, and thus the capacity of the helper virus to rescue itself as it propagates. This vector has been previously used to clone the full-length murine dystrophin cDNA (13.8 kb) under the control of the murine muscle specific creatinine kinase (6.5 kb) and a CMV promoter - E.coli LacZ gene cassette (4.6 kb) (Kochanek et al., 1996). This completely debilitated, recombinant virus propagated efficiently in 293 cells (which complement E1 functions) in the presence of a helper mutant Ad virus (SV5). The yield after cesium chloride density gradient banding was $\sim 5 \times 10^9$ pfu obtained from 1.4×10^8 293 cells with about 1% contamination of helper virus as determined by a plaque forming unit (pfu) assay on 293 cells and by southern blot analysis. The recombinant HD vector was efficient in co-expressing the dystrophin protein and β -gal in primary myoblasts derived from mdx mouse (a genetic and biochemical model for human DMD disease) and *in vivo* (Kochanek et al., 1996, Clemens et al., 1996).

In a more recent study we delivered the leptin cDNA using the HD virus, testing the hypothesis that elimination of the viral protein coding sequences would diminish the vector's cellular immunogenicity and toxicity, and hence support its longevity *in vivo* (Morsy et al., 1998a-c). Since both the viral proteins and the transgene were factors implicated in the cellular immunogenicity of recombinant Ad viruses, we designed experiments to compare the HD and Ad vectors in ob/ob mice that are naive to leptin (in which the protein is potentially immunogenic), as well as in lean mice that normally express leptin.

In this study, we showed that HD-leptin provided greater safety as reflected by absence of liver toxicity, cellular infiltrates, extended longevity of gene expression and stability of vector DNA in livers of treated mice over that observed with 1st generation Ad-mediated leptin treatment.

III. Safety of HD compared to 1st generation ad vectors

Mice were treated with a single tail intravenous infusion of $1-2 \times 10^{11}$ particles of either HD-leptin, Ad-leptin, control Ad- β -gal vector or an equal volume of control buffer. Toxicity was evaluated by measuring the levels of released liver enzymes in sera and by studying the histopathology of liver sections obtained from treated animals at successive intervals post treatment. **Figure 1** shows the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the sera of lean mice at one, two and four weeks post-treatment (similar results were observed in treated ob/ob mice - data not shown). Liver toxicity, as reflected by the significant elevation in AST and ALT serum levels over basal control levels, was observed only in mice treated with Ad- β -gal and Ad-leptin, but not HD-leptin. Ad-vector-associated toxicity observed in both the lean and ob/ob treated mice was most significant at one week, was present but to a less significant extent at two weeks, and was resolved by four weeks post-treatment. In contrast, HD-treatment was not associated with liver toxicity as reflected by the AST and ALT serum levels which were essentially indistinguishable from controls.

Liver sections of HD-leptin-treated lean mice were histologically indistinguishable from control liver sections at all time points tested post treatment (**Table 1**). In contrast, Ad-leptin and Ad- β -gal treated mice displayed hepatic pathology (hepatopathy) throughout the first 1-2 weeks post-treatment, which resolved by week 4.

IV. Efficacy of HD compared to 1st generation Ad vectors

In the lean mice, treatment with Ad-leptin resulted in weight loss that lasted for only 7-10 days which was associated with a transient increase in serum leptin levels (**Figure 2A** and **B**). In contrast, treatment with HD-leptin resulted in approximately 20% weight loss that persisted at least two months and high serum leptin levels (6- to 10-fold over background) (**Figure 2A** and **B**). Weight loss in HD-leptin-treated mice was associated with satiety that persisted over a longer period (2-3 weeks) than in those treated with Ad-leptin (5-7 days) (**Figure 2C**). Vector DNA in the livers of Ad-leptin treated mice was rapidly lost and fewer than 0.2 copies per cell were detected, compared to 1-2 copies per cell following HD-leptin treatment at 8 weeks post-injection (data not shown). These effects can be correlated with the duration of gene expression obtained with these two vector types. Gene expression mediated by Ad-leptin was transient and almost undetectable as early as 1 week post treatment as seen by northern blot analysis of total liver RNA, whereas that mediated by HD-leptin persisted for at least eight weeks (data not shown).

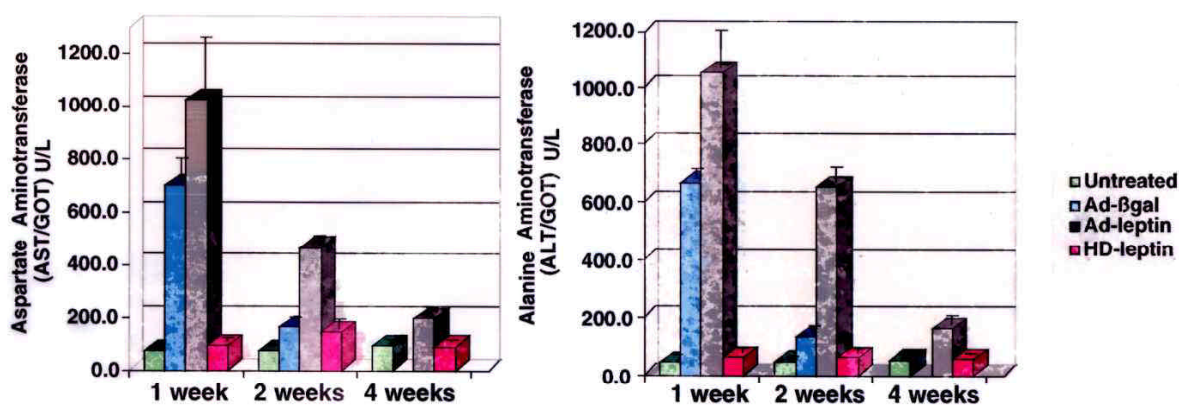


Figure 1. Mice were treated with Ad- β -gal, Ad-leptin, HD-leptin or dialysis buffer (controls). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the sera of lean control and treated mice are plotted at one, two and four weeks post-treatment.

Table 1. Table 1 is a summary of the liver histopathology findings. Histopathology refers to the displayed degenerative hepatic pathology found in livers of Ad-leptin and Ad- β -gal treated mice. This hepatic pathology was characterized by foci of round cell infiltration composed almost entirely (> 98%) of T-cells, individual liver cell necrosis, increased liver cell mitotic activity, and dissociation of hepatic cords. At two weeks post-treatment, Ad-leptin treated mice display a similar, but less pronounced hepatic pathology. The cellular infiltration observed resolved by four weeks post-treatment; there was almost an absence of lesions in the Ad-leptin treated mice, with only a trace of individual cell death present, which is within normal ranges. Examination of liver sections obtained from ob/ob mice reflected similar Ad-vector associated histopathology. Liver histology was indistinguishable between HD-leptin-treated and untreated control mice.

Liver Toxicity : Histopathology

Lean liver	1 week	2 weeks	4 weeks
■ Untreated	Not remarkable	Not remarkable	Not remarkable
■ Ad- β gal	Hepatopathy	Hepatopathy very slight	Not remarkable
■ Ad-leptin	Hepatopathy	Hepatopathy very slight	individual cell necrosis (trace)
■ HD-leptin	Not remarkable	Not remarkable	Not remarkable

The ob/ob mice are naive to leptin and thus transgene immunogenicity is not an unexpected finding. In these animals, similar to what was observed in the lean mice, HD-leptin was found to be more effective than the first-generation Ad-leptin vector. In the ob/ob mice treated with Ad-leptin, transient body weight loss of ~25%, followed by weight gain, two weeks after treatment was observed (**Figure 3A** and **B**). Associated, serum levels of leptin increased only for a short period during the first 4 days of treatment, returning to baseline levels within ten days post-injection (**Figure 3C**). Similar to the results obtained in lean mice, the Ad-leptin vector DNA (data not shown) was also rapidly lost (< 0.2 copies per cell were detected by 2 weeks post treatment, and undetectable by 8). In contrast, the ob/ob HD-leptin-treated mice had increased serum leptin levels up to ~15 days post-treatment, after which the levels gradually dropped to baseline over the subsequent 25 days (**Figure 3C**). The initial rise in leptin levels correlated with rapid weight reduction resulting in > 60% weight loss (reaching normal lean weight) by one month (**Figure 3A**). Weight loss was maintained for a period of 6-7 weeks post-treatment. The overall HD-leptin-mediated prolonged effect was also reflected in the accompanying phenotypic correction, which lasted longer than that seen in litter mates treated with Ad-leptin (6-7 versus 2-3 weeks) (**Figure 3B**). As leptin levels dropped to baseline (**Figure 3C**), a gradual increase in body weight was observed (**Figure 3A**). Satiety was observed in association with increased leptin levels, and appetite suppression was sustained for a longer period (~1 month) compared to the short transient effect induced by Ad-leptin (~10 days) (**Figure 3D**). Leptin-specific antibodies were

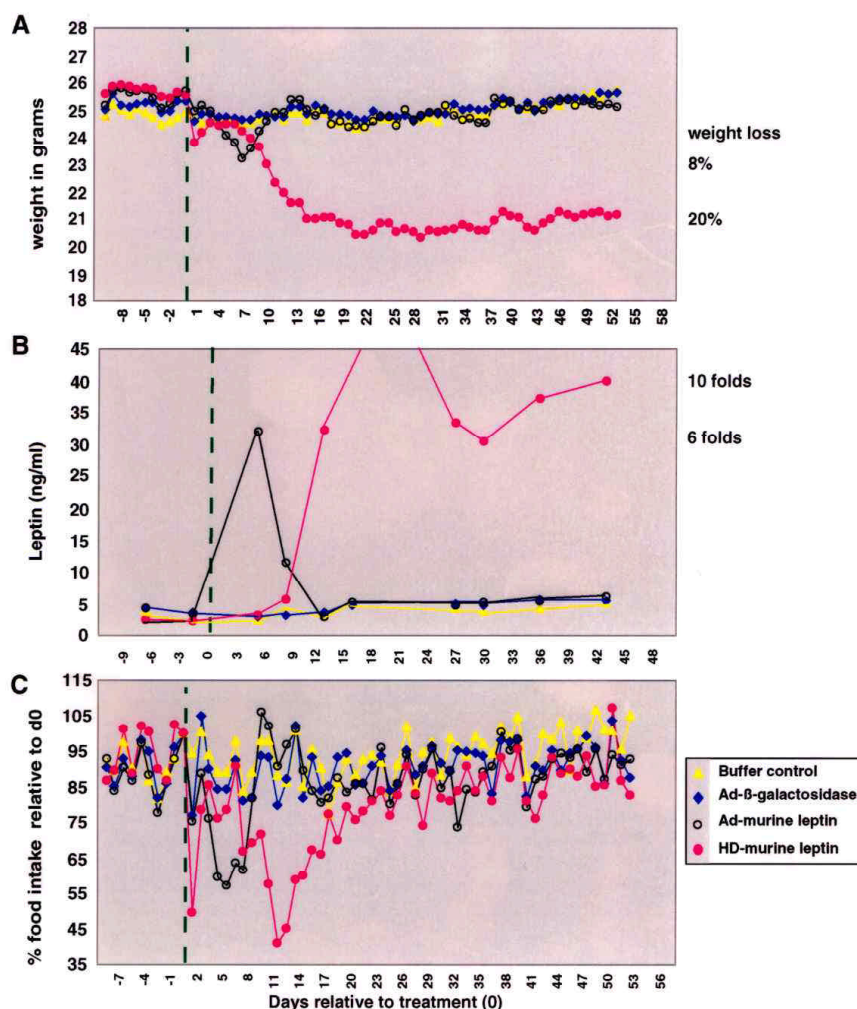
detected in the sera of ob/ob Ad-leptin- and HD-leptin-treated mice (data not shown) suggesting immunogenicity of leptin in these naive animals. Results of southern blot analysis showed greater stability of HD-vector DNA over Ad-vector DNA in livers of ob/ob treated mice compared at similar time points, the analysis revealed eventual loss of the HD-vector DNA over the 8 week time interval (data not shown). Approximately 75% less vector DNA was detected in the livers of HD-leptin-treated ob/ob mice at 4 and 8 weeks post-treatment compared to the persistent levels found in the livers of HD-leptin-treated lean litter mates at similar time points (data not shown). Gene expression in ob/ob Ad-leptin-treated mice correlated with the DNA findings, RNA levels were below the sensitivity level of detection at one week post-treatment, whereas in HD-leptin-treated mice, gene expression was detected up to four weeks post-injection and was undetectable at eight weeks (data not shown).

V. Future prospects for gene therapy: regulation of gene expression

Development of gene therapy vectors allowing ligand-inducible control of therapeutic gene expression will surely impact the safety and efficacy of future gene therapy protocols. Inducible systems capable of modulating gene expression in a ligand-dependent manner are being tested in a variety of different viral vectors with increasing frequency.

Regulatable systems consist of a chimeric transactivator and its inducible promoter. In the examples which follow, the transactivators contain the following constituents:

Figure 2. HD-leptin and Ad-leptin effects in lean mice. Animals were injected via the tail vein with a single dose of $1-2 \times 10^{11}$ particles of HD-leptin (n=5), Ad-leptin (n=10), Ad- β -gal (n=10), or the equivalent volume of dialysis buffer (n=10). The time course shows (A) weight (grams) and the percent of maximum weight loss observed in Ad-leptin and HD-leptin treated mice (8% and 20%, respectively); (B) serum leptin levels, collected 2-3 times weekly (ng/ml) and the maximum fold increase in serum leptin levels above basal observed in the Ad-leptin and HD-leptin treated mice (6 fold and 10 fold, respectively); (C) the percentage of food intake relative to untreated control mice. The dashed line marks day 0 relative to day of injection.



(i) functional element(s) which interact with a small molecule inducing compound; (ii) a DNA-binding domain which does not exhibit cross reactivity with endogenous cellular sequences; and (iii) a transactivation domain. The inducible promoter consists of a minimal promoter (or TATA box) linked downstream to repeats of the transactivator recognition sequence. In the presence of inducer, the chimeric transcription factor should bind specifically to its DNA recognition sequence within the inducible promoter to transactivate target gene expression. Below is a brief review of the performance of the most promising drug-inducible gene expression systems in viral vectors.

VI. Tetracycline-inducible system

A regulatable gene expression system utilizing the bacterial tetracycline repressor protein was originally described by Gossen and Bujard (1992). The tetracycline repressor was fused to a viral transactivation domain to form a tetracycline-controlled transactivator (tTA). This chimeric protein could activate transcription in mammalian cells from an inducible promoter consisting of tetracycline operator sequences fused to a minimal promoter. In the presence of tetracycline, protein-ligand interactions produced a conformational change in tTA so that it could no longer bind operator sequences and activate transcription, thus making this a tetracycline-repressible system.

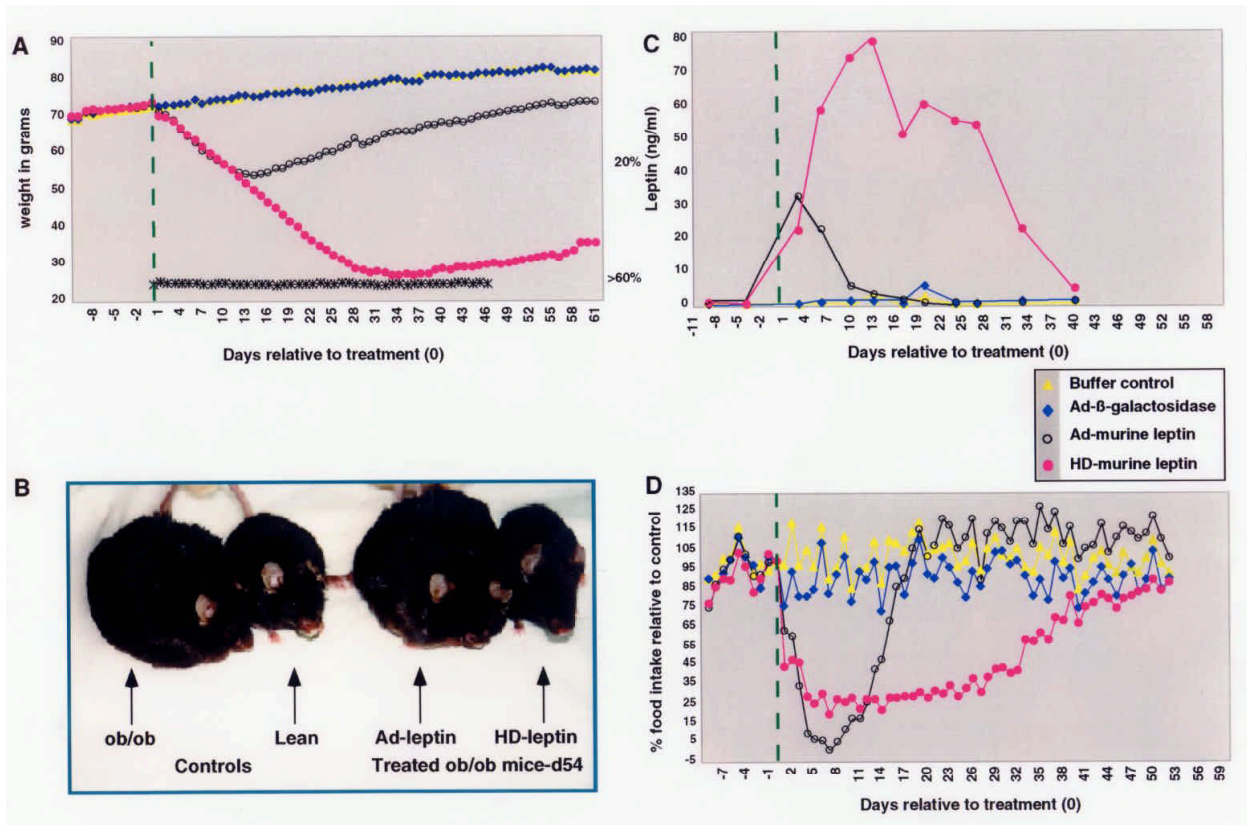


Figure 3. HD-leptin and Ad-leptin effects in ob/ob mice. Essentially as described in Fig. 2, animals were injected in the tail vein with a single dose of $1-2 \times 10^{11}$ particles of HD-leptin (n=5), Ad-leptin (n=10), Ad-β-gal (n=10), or the equivalent volume of dialysis buffer (n=10). Lean control values are plotted for comparison. The time course shows (A) weight (grams) and the percent of maximum weight loss observed in Ad-leptin and HD-leptin treated mice (20% and 60%, respectively); (B) Phenotypic correction of HD-leptin-treated ob/ob mice. On the left is a representative ob/ob mouse treated with HD-leptin at day 54 post-treatment, next to a litter mate treated with Ad-leptin. The Ad-leptin-treated mouse initially lost weight during the first two weeks following the treatment, and subsequently gained weight. At 54 days post-Ad-leptin treatment, ob/ob mice are indistinguishable from untreated ob/ob control litter mates, whereas HD-leptin-treated mice remained indistinguishable from untreated lean control mice. Untreated ob/ob and lean control mice are shown for comparison as labeled. (C) serum leptin levels, collected 2-3 times weekly (ng/ml); (D) the percentage of food intake relative to untreated control mice. The dashed line marks day 0 relative to day of injection.

Figure 4. Reverse tetracycline-regulated gene expression. The reverse tetracycline-controlled transactivator (rtTA) consists of the reverse tetracycline repressor (rtetR) fused to a VP16 transactivation domain. In the presence of doxycycline (Dox), rtTA binds and transactivates transcription from the inducible promoter consisting of seven tet operator sites (tetO) located upstream of the human cytomegalovirus (hCMV) immediate early minimal promoter.

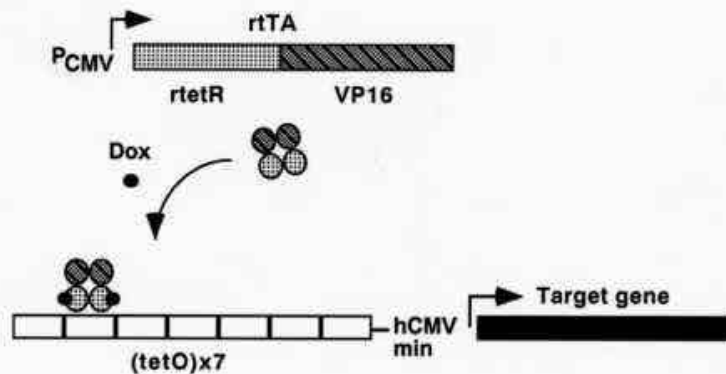
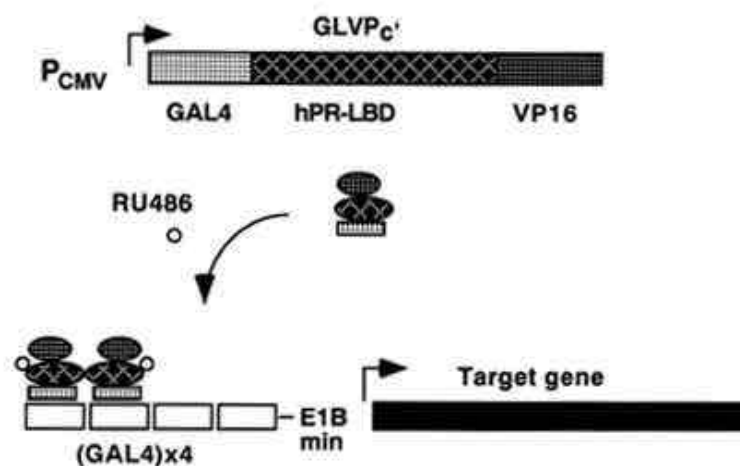


Figure 5. Antiprogestin-regulated gene expression. The optimized transactivator GLVPC' consists of a yeast GAL4 DNA-binding domain fused to a truncated human progesterone receptor ligand binding domain (hPR-LBD) and a VP16 transactivation domain. In the presence of RU486, GLVPC' binds to an inducible promoter consisting of four GAL4 DNA-binding sites fused to the adenovirus E1B minimal promoter to transactivate gene expression.



The repressible system was later modified to an inducible system following the isolation of tetracycline repressor mutants conferring a reverse phenotype (Gossen et al., 1995). In contrast to the wild type protein, the reverse tetracycline repressor required tetracycline or a derivative such as doxycycline to bind operator sequences. Correspondingly, the reverse tetracycline transactivator (rtTA) could now activate gene expression in the presence of drug, rendering the system more suitable for therapeutic applications (Figure 4).

Several retroviral vectors designed to evaluate tetracycline-inducible control of gene expression have been described (Bohl et al., 1997; Lindemann et al., 1997; Watsuji et al., 1997). Doxycycline-regulated control of erythropoietin (Epo) secretion was maintained for a period of 5 months in immunocompetent mice receiving implants of primary myoblasts transduced by a retroviral vector carrying rtTA and inducible Epo (Bohl et al., 1997). Although basal transcription under uninduced conditions was observed, overall induction of Epo secretion increased 70-fold. In similar cell implantation experiments, Lindemann et al. (1997) reported significant induction of doxycycline-regulated human growth hormone secretion in C3H mice for 46 days. Basal activity from the inducible promoter was apparent directly after implantation, but decreased over time. In vitro, an 1800-fold induction of chloramphenicol acetyltransferase (CAT) activity in response to doxycycline administration was observed in coinfection experiments utilizing recombinant adenoviral vectors expressing rtTA and an inducible CAT reporter construct (Molin et al., 1998). In vivo, intramuscular injection of these vectors into immunocompetent mice gave high levels of induction of CAT expression with variable basal activity.

Comparable results were recently achieved using a recombinant AAV vector expressing rtTA and inducible Epo (Bohl et al., 1998). A single intramuscular injection of AAV into normal mice was sufficient to sustain Epo expression in a doxycycline-dependent manner over a 29-week period. Serum Epo levels were approximately 10-fold higher than basal concentrations.

VII. Antiprogestin-regulated gene switch

The human progesterone receptor (hPR) is a member of the nuclear hormone receptor superfamily whose functional ligand binding domain (LBD) has been used to inducibly regulate gene expression. Original hPR-based systems utilized a human progesterone receptor with a truncated ligand binding domain (Wang et al., 1994; Delort and Cappechi, 1996). While the truncated receptor is no longer able to bind its natural ligand progesterone, it retains the ability to bind progesterone antagonists such as RU486 (Garcia et al., 1992; Vegeto et al., 1992). In developing an inducible system, a chimeric transactivator consisting of a viral transactivation domain fused to a yeast GAL4 DNA-binding domain and the truncated hPR LBD was constructed. The inducible promoter was composed of a series of GAL4 recognition sequences upstream of the adenovirus E1B TATA box.

Subsequent modifications to the transactivator have increased the transcriptional activation potency and sensitivity of the system to inducing compound (Wang et al., 1997). Extension of the ligand binding domain and repositioning of the transactivation domain to the carboxy terminus produced an optimized transactivator capable of activating target gene expression at RU486 concentrations as low as 0.01nM (Figure 5).

Performance of the antiprogesterin-regulated system has recently been tested in adenovirus and herpes simplex virus vectors. *In vitro*, approximately 600-fold induction of gene expression in response to RU486 was observed upon coinfection of cells with recombinant adenoviral transactivator and reporter vectors (Molin et al., 1998). *In vivo*, stereotactic injection of herpes simplex virus vectors containing the transactivator and an inducible lacZ reporter gene into rat hippocampus produced 150-fold activation of reporter expression following *i.p.* administration of RU486 (Oligino et al., 1998).

VIII. Dimerization-based gene regulation

Development of a regulatable system based on chemical inducers of dimerization (CIDs) stems from earlier studies delineating the mechanism of action of immunosuppressant compounds such as FK506, rapamycin, and cyclosporin A. These drugs functionally inhibit signalling pathways affecting T-cell activation and proliferation by mediating the dimerization and inactivation of endogenous cellular proteins involved in these processes.

Rivera et al. (Rivera et al., 1998) have employed a dimerization-based strategy to develop a humanized system for inducing gene expression *in vivo*. In this system, a transcriptional transactivation domain is provided by the carboxy-terminal region of the NF B p65 protein, which is

fused to the rapamycin-binding domain of FKBP12-rapamycin associated protein (FRAP). The DNA-binding domain, termed ZFHD1, is a composite zinc finger-homeodomain chimeric protein with novel DNA recognition specificity (Pomerantz et al., 1995) fused to a series of three repeats of the cellular protein FKBP12. These two proteins dimerize in the presence of rapamycin to form a functional transactivator which binds an inducible promoter containing ZFHD1 binding sites upstream of an hCMV minimal promoter (Figure 6).

Although reports demonstrating the utility of such a system in viral vectors have not yet been published, the rapamycin-inducible system has been used to regulate hGH secretion in a cell implantation model in nude mice (Magari et al., 1997). Upon administering rapamycin to nude mice implanted with cells stably expressing hGH under control of the inducible promoter, peak levels of hGH secretion were observed within 24 hours with no detectable basal activity. Overall induction levels were found to be dependent on a number of parameters, including the dose of rapamycin received.

IX. Conclusions

The leptin model used in the studies described above have provided a very instructive animal model to investigate

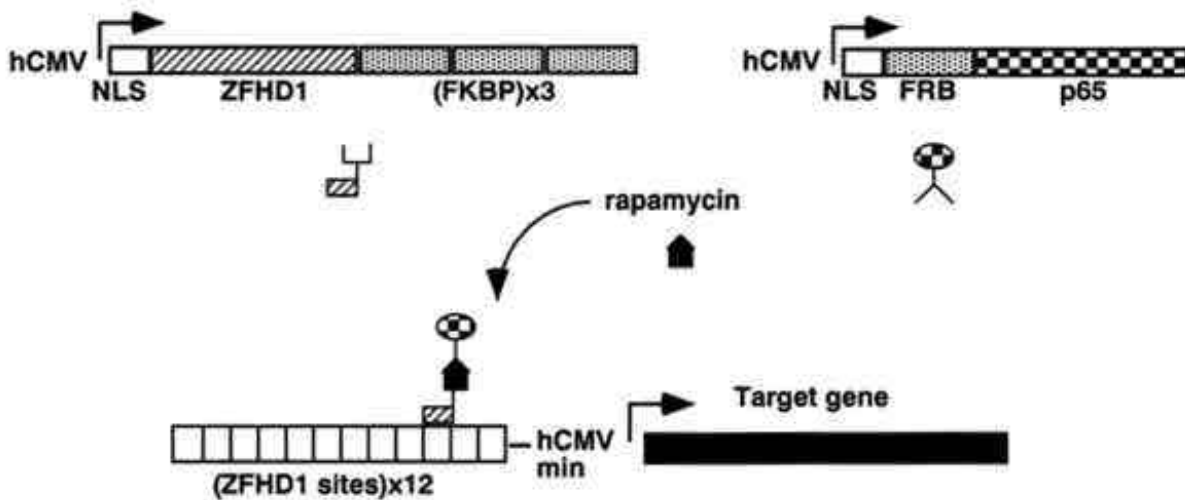


Figure 6. Rapamycin-regulated gene expression. The DNA-binding domain (ZFHD1) of the functional transactivator is a composite zinc finger/homeodomain chimera fused to three FKBP12 repeats (FKBP). The transactivation domain consists of the carboxy-terminal portion of the NF B p65 protein (p65) fused to the FKBP12-rapamycin binding (FRB) domain from the cellular protein FRAP. NLS denotes nuclear localization signals. In the presence of rapamycin, DNA-binding and transactivation domains dimerize via FKBP and FRB to transactivate gene expression from an inducible promoter consisting of twelve ZFHD1 binding sites and an hCMV minimal promoter.

the influence of both vector design and transgene product on the duration of expression after gene transfer. The HD-vector system is a significant advance over existing Ad vectors with regards to safety, vector-mediated immunogenicity and insert capacity (up to 37kb). In addition to the gain of these valuable properties, the HD-vectors have not lost the features that contributed to the general attractiveness of Ad vectors which include: (i) efficient *in vivo* gene delivery, and (ii) high titer production. This system has come a long way in terms of development and ease of vector preparation and purification. Several studies involving the development of helper-dependent vectors were hindered by the complexity of the system (Mitani et al., 1995, Kochanek et al., 1996, Kumar-Singh and Chamberlain, 1996, Hardy et al., 1997, Lieber et al., 1996).

The characterization of size requirements for efficient packaging and the generation of crippled helper viruses greatly enhanced the prospects of these new vectors in becoming a promising tool for gene delivery (Parks and Graham, 1997, Parks et al., 1996). Further modifications and fine tuning are required to convert the HD vector system to an industrially scaleable system for clinical utility.

Regarding the potential for drug-inducible gene expression in viral vectors, a number of requirements will ultimately need to be fulfilled before regulated systems such as those described here are included in human gene therapy protocols. First, components of the system (including the transactivator, inducible promoter, and inducing compound) should not interfere with normal cellular processes. Second, a strong induction profile of therapeutic gene expression in the presence of drug coupled with low basal activity in the uninduced state will be important for general applicability of the system. Third, the inducer will preferably be an orally bioavailable, physiologically inert compound that is cleared from body tissues within a reasonable length of time. Finally, due to potential host immune recognition, the transactivator protein(s) should ideally be non-immunogenic. This is particularly important in cases where long-term correction of a genetic deficiency is desired. Although no inducible gene regulation system can claim to meet all of these requirements thus far, their continued evaluation in the context of gene therapy vectors will hopefully yield optimized systems capable of making exogenous control of gene regulation a reality.

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