

Promoters and Control Elements: Designing Expression Cassettes for Gene Therapy

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Abstract: It has become apparent that the clinical success anticipated in the field of gene therapy has been limited by progress in several of the fundamental areas of genetics, molecular and cellular biology relevant to its application. Whilst a great deal of effort has been made in the evaluation of transgenes, it is only more recently with the advance of vector systems that attention has begun to be focused upon the means and control of transgene expression. Until recently, the majority of constructs have employed ubiquitous viral promoters to drive expression from simple gene expression cassettes using viral promoters and lacking introns, 3' untranslated regions (UTRs), locus control regions (LCR's), matrix attachment regions (MAR's) and other such genetic components. It has consequently emerged that these elements may have a key role in determining the levels and longevity of gene expression attainable *in vivo*, irrespective of the vector system utilised. The majority of gene therapy applications would also benefit from the specific optimisation of 'tailor-made' expression cassettes to optimise their therapeutic efficacy. In conjunction with modification of vector tropism and strategies to limit their immunogenicity, this should create vectors suitable for the clinical application of gene therapy. This review aims to highlight some of the principle considerations of gene expression *in vivo*, and the means by which it may most effectively be achieved, whether this is via the minimal modification of an existing eukaryotic promoter or by the more extensive design of a novel promoter and associated elements.

Keywords: promoters, tissue-specific, transgene, transcription regulation, expression cassette.

INTRODUCTION

In recent years gene therapy has begun to make the advance from proof of concept to proof of practice, but the primary obstacle to its clinical utility remains the inability of current vector systems to safely and efficiently deliver transgenes to target tissue *in vivo* [Somia *et al.*, 2000]. Whilst considerable progress has been made in the development of both viral and non-viral vectors, the goal of systemic administration for safe and specific target tissue transduction remains some way off. Applications suited to local delivery would similarly benefit from an improved ability to target tissue in an efficient and selective fashion. Both situations would derive advantage from a reduced risk of ectopic gene expression. Most gene therapy protocols have utilised vectors that retain their wild-type tropism (if any) and employ simple transgene expression cassettes driven by strong viral promoters. Such generic vectors have been popular because of their broad utility, and have typically proved extremely powerful *in vitro* and in some pre-clinical models *in vivo*. More recently it has become clear that modification of vector tropism to either expand or restrict gene delivery to specific tissues *in vivo* (transductional targeting), and of vector expression cassettes to add further tissue selectivity (transcriptional targeting), may yield significant advantages in gene therapy and serve to minimise the input titer required to evoke a phenotypic

response *in vivo*. Control of transgene expression may also be extended to utilise elements that respond to particular stimuli, such as blood glucose levels, or regulable on/off switches that permit stringent exogenous control of transgene expression. Increased understanding of the molecular mechanisms of eukaryotic transcription has also begun to permit the enhancement of expression levels from otherwise weak eukaryotic promoters by the incorporation of elements such as introns, matrix attachment regions (MAR's), and locus control regions (LCR's) into complex expression cassettes that are more homologous to the natural chromosomal organisation of genes.

VIRAL AND EUKARYOTIC PROMOTERS

Viral Promoters

The majority of studies in gene therapy research to date have utilised viral promoters. In their natural context strong viral promoters are required for efficient viral propagation, and they frequently induce much higher levels of transcription than eukaryotic promoters by using mechanisms to control and recruit host transcription machinery. Moreover, by necessity they tend to be far more compact and hence easier to manipulate and accommodate into gene therapy vectors. The utilisation of the ubiquitous cytomegalovirus immediate early (CMV-IE) promoter and enhancer has proven to be particularly popular in gene transfer vectors because of its ability to drive very high levels of transgene expression in many tissue types. Other viral promoters in use include the simian virus 40 (SV40),

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Rous sarcoma virus long terminal repeat (RSV-LTR), Moloney murine leukaemia virus (MoMLV) LTR, and other retroviral LTR promoters. The use of viral promoters has thus been widespread and successful *in vitro* and for certain applications *in vivo*. Notably, pre-clinical studies for both haemophilia A and haemophilia B have demonstrated therapeutic and sustained levels of transgene expression of a year or more from a variety of vectors utilising viral promoters. Vanden Driessche *et al.* [Van Den Driessche *et al.*, 1999] and Snyder *et al.* [Snyder *et al.*, 1999] utilised LTR-driven retroviral and adeno-associated viral (AAV) constructs respectively; the former study demonstrating expression of factor VIII for over a year in mice (4 of 13 mice expressed FVIII at physiologic or greater levels of 500-12500 mU/ml), and the latter a similar period of factor IX expression in both mice and dogs (at up to 2 µg/ml in mice and up to 100 ng/ml in dogs; ~5 µg/ml is physiological). Herzog *et al.* [Herzog *et al.*, 1999] used a CMV-IE driven AAV vector to express factor IX for at least 17 months in dogs (at 3-70 ng/ml).

Unsurprisingly however, eukaryotic cells have evolved mechanisms to detect and silence viral transgene expression. Viral promoters have manifested a frequent inability to sustain transgene expression *in vivo*, but despite considerable evidence that viral promoters are prone to inactivation and silencing *in vitro* and *in vivo*, a large proportion of gene therapy applications continue to utilise them to drive transgene expression. The CMV promoter has been commonly shown to be outlasted by many eukaryotic constructs *in vivo*, and despite incorporating four NF- κ B response elements [Pan *et al.*, 1999], has in many instances been shown to be specifically downregulated by tumour necrosis factor (TNF) and interferons (IFNs) like other viral promoters, such as SV40 and LTRs [Acsadi *et al.*, 1998; Ghazizadeh *et al.*, 1997; Harms *et al.*, 1995; Qin *et al.*, 1997]. Retroviral LTRs are subject to additional complex silencing mechanisms [Alur *et al.*, 2002]. Moreover, it has become clear that viral promoters are inherently prone to this silencing effect *in vivo*.

A recent study using high-capacity adenoviral (HCAd) vectors also suggested that CMV promoter-induced transgene expression was more prone to invoke an immune response to the transgene product, compared to a vector driven by the phosphoglycerate kinase (PGK) promoter, which is also non-specific [Schiedner *et al.*, 2002]. This may be because CMV is able to drive higher levels of transgene expression in antigen-presenting cells than PGK, and so more efficiently primes an immune response to the transgene product. Induction of anti-transgene immunity has also been observed with AAV vectors using CMV or chimeric CMV-LTR promoters, with the severity being dependent upon the route of administration, transgene, and strain of mouse [Cordier *et al.*, 2001; Ge *et al.*, 2001; Song *et al.*, 1998]. The poor efficiency with which AAV vectors transduce mature dendritic (antigen-presenting) cells may be offset by the activity of CMV in immature antigen-presenting cells *in vivo* [Cordier *et al.*, 2001]. It is certainly clear that powerful non-specific promoters are more prone to induce anti-transgene immunity, and that specific eukaryotic promoters may be used to avoid this effect.

Viral Promoters in Adenoviral Vectors

The evolution of adenoviruses (Ads) as vectors for gene therapy has revealed some of the limitations of viral promoters such as CMV-IE, and the necessity to develop genomic eukaryotic alternatives. The inability of first or second generation adenoviral vectors to sustain long-term transgene expression *in vivo* has been primarily attributed to adaptive immune-mediated elimination of virally transduced cells due to latent expression of viral genes or immunogenic transgenes [Dai *et al.*, 1995; Lusky *et al.*, 1998; Yang *et al.*, 1996], as well as an innate response that is not dependent on viral gene expression [Adesanya *et al.*, 1996; Lieber *et al.*, 1997; Otake *et al.*, 1998]. The development of adenoviral vectors deleted for all viral genes, known alternately as 3rd generation (helper-dependent, gutted or high-capacity vectors (HCAd's); reviewed in [Kochanek, 1999]), has not wholly overcome this because systemic administration of HCAd vectors is still likely to induce an innate response that may exacerbate silencing of viral promoters *in vivo* [Ehrhardt *et al.*, 2002; Muruve *et al.*, 1999; Schnell *et al.*, 2001; Zhang *et al.*, 2001]. This innate response has been demonstrated to lead to suppression of the CMV promoter via the induction of TNF and IFN prior to the onset of any adaptive immune response *in vivo* [Sung *et al.*, 2001]. This effect on viral promoters has been corroborated by numerous reports. Upon administration of an HCAd, Chen *et al.* [Chen *et al.*, 1997] observed a disproportionate fall in β -galactosidase transgene expression levels by 84 days compared to the persistence of vector DNA in *lacZ* transgenic mice, implying suppression of the CMV promoter, a phenomenon that has also been observed by others [Ehrhardt *et al.*, 2002; Yao *et al.*, 1996]. Whilst Morsy *et al.* [Morsy *et al.*, 1998] were able to demonstrate expression of leptin from a CMV driven HCAd for at least 2 months *in vivo* in lean mice and up to a month in obese (leptin deficient) mice, other researchers have observed transgene expression for periods of up to a year or more utilising third-generation vectors incorporating eukaryotic promoters. Similar discrepancies have been seen comparing viral and eukaryotic promoters in first-generation Ads.

Viral Promoters in AAV Vectors

These observations have not been limited to results with episomally maintained Ad vectors. Several studies have directly compared the ability of CMV and eukaryotic promoters to drive transgene expression *in vivo* using AAV vectors (that potentially integrate into the host genome). Cordier *et al.* [Cordier *et al.*, 2001] demonstrated the limitations of using CMV in both first generation Ad and AAV vectors delivered by intramuscular injection to express α -sarcoglycan in a mouse model of muscular dystrophy. In this study a muscle creatine kinase (MCK) promoter-driven AAV produced α -sarcoglycan for up to a year to therapeutic effect, whilst a CMV promoter-driven counterpart declined significantly by 3 months. Similarly, the MCK promoter-driven Ad produced detectable levels of transgene expression for over 16 weeks, whilst CMV-induced vector declined by 4 weeks. Nakai *et al.* [Nakai *et al.*, 1998] also reported factor IX expression at up to 3 µg/ml for at least six months from a systemically administered AAV utilising the elongation factor 1 (EF1) promoter, whilst CMV promoter-induced

expression of up to 0.1 µg/ml had declined to background by 5 weeks despite the persistence of viral DNA, indicating CMV promoter shutoff.

Retroviral LTR Promoters

Retroviral vectors have been successfully used for long term application in clinical studies where gene transfer confers functional selectivity on the transduced cell population [Cavazzana-Calvo *et al.*, 2000]. Regardless, a large proportion of integrating retroviral vectors are immediately silenced by the heterochromatic packaging of viral DNA (known as position effect variegation). Without selection, they are particularly prone to transcriptional silencing following transduction and integration *in vivo* [Pannell *et al.*, 2001; Sabatino *et al.*, 2000]. Viruses that escape this effect may be subsequently silenced following transient expression of viral genes by poorly understood mechanisms, including the methylation of C G dinucleotide pairs [Challita *et al.*, 1994; Halene *et al.*, 1999; Pannell *et al.*, 2001]. Challita *et al.* [Challita *et al.*, 1994] correlated the silencing of a retrovirus vector in serially transplanted haemopoietic stem cells *in vivo* with the increased methylation of integrated provirus: LTR-induced expression was high in primary transduced stem cells, but absent in over 90% of secondary transduced stem cells and completely absent in tertiary cells. This silencing correlated with a 3-4 fold increase in methylation-sensitive restriction of proviral LTRs in secondary and tertiary stem cells. Suppression of parasitic DNA sequence elements is now thought to be a primary function of cytosine methylation [Wolffe *et al.*, 2000; Yoder *et al.*, 1997]. Other groups have demonstrated that eukaryotic promoters, such as the human α_1 -antitrypsin (hAAT) and murine RNA polymerase II (large subunit) promoters, greatly exceed the level of LTR-driven expression from retrovirus vectors *in vivo* [Okuyama, 1996; Rettinger *et al.*, 1994].

There is additional evidence for the involvement of other silencing mechanisms that are effected via 'silencing elements' in the viral LTRs, involving the recruitment of histone deacetylase and other chromatin remodelling complexes that function prior to the methylation of viral DNA [Alur *et al.*, 2002] (reviewed in [Pannell *et al.*, 2001]). Viral LTR promoters have also been shown to be suppressed by TNF and IFN *in vitro* [Qin *et al.*, 1997], and interferons have been shown to suppress retroviral transgene expression by over 75% *in vitro* [Ghazizadeh *et al.*, 1997]. This latter study demonstrated a posttranscriptional block in expression connected with the retroviral transcript 3'UTR, and hence implied the possibility of avoiding this through the modification of the 3'UTR and polyadenylation signals. Hejnar *et al.* [Hejnar *et al.*, 2001] utilised a CpG island from a mouse housekeeping gene to protect the RSV-LTR promoter from the silencing effect of *de novo* methylation of the viral promoter. Notably this effect was dependent on the presence of three Sp1 sites in the CpG island suggesting factors recruited by these sites may play a key role in avoiding heterochromatic silencing of promoters. Other groups have modified or deleted large parts of the viral LTRs to improve safety and remove sequences associated with viral silencing, generating self-inactivating (SIN) vectors that demonstrate greatly improved expression profiles [Alur

et al., 2002; Lotti *et al.*, 2002; Zufferey *et al.*, 1998]. Having previously observed retroviral silencing in over 95% of MoMLV transduced cells, Prasad Alur *et al.* [Alur *et al.*, 2002] recently demonstrated that deleting negatively acting *cis* elements in the viral LTRs, retaining a permissive primer binding site (PBS) sequence and using the eukaryotic -glucuronidase promoter could overcome this downregulation *in vivo* in a mouse model of lysosomal enzyme deficiency. Another study demonstrated that the use of alternative optimised LTR promoters to drive expression from retroviral vectors may avoid severe silencing effects in the liver *in vivo* [Ohnishi *et al.*, 2002]. Further mechanisms of avoiding the silencing of viral promoters, such as the use of LCRs and MARs, are discussed later.

Viral Promoters in Plasmid Vectors

Systemically administered plasmid vectors can also induce hepatotoxicity and both humoral and cell-mediated immune responses that limit their application *in vivo*, an effect most frequently observed when viral promoters such as CMV are used to drive transgene expression [Loisel *et al.*, 2001; Tousignant *et al.*, 2000]. Studies have indicated that inflammation associated with plasmid DNA may be due to the presence of particular unmethylated C G dinucleotides ('CpG-S DNA') (reviewed in [Krieg, 1999]). These arise at the expected mathematical frequency in prokaryotic DNA, but at only 1/5th of the expected frequency in the vertebrate genome where they are frequently methylated. Some of these are concentrated in the upstream regulatory regions of genes (commonly housekeeping genes) in clusters of >200bp, where they are termed 'CpG islands' and are frequently undermethylated, but not immunostimulatory ('CpG-N DNA') [Jones, 1999; Krieg, 1999]. Methylation of these upstream islands is thought to constitute a key heritable mechanism of negative developmental gene regulation. Discrimination of high-frequency non-methylated C G dinucleotides in parasitic DNA by host cell machinery triggers a considerable inflammatory response. A similar effect has been observed with HCAAd vectors using large amounts of prokaryotic lambda stuffer DNA [Parks *et al.*, 1999]. Reducing the C G content of plasmid vectors (including the CMV promoter which contains ~75 of these pairs) reduced the induction of pro-inflammatory cytokines and dramatically improved transgene expression profiles from both intravenously and intranasally administered plasmid vectors [Yew *et al.*, 2001; Yew *et al.*, 2002]. These studies did not directly evaluate the effect of reducing the C G content of the CMV promoter alone, and it remains to be seen whether a C G depleted or modified CMV promoter/enhancer demonstrates improved persistence in other vector systems.

EUKARYOTIC PROMOTERS

Introduction

It is emerging that the use of strong eukaryotic promoters may prove highly advantageous in achieving long-term expression *in vivo*. Moreover, the use of a cell-selective eukaryotic promoter adds an additional layer of specificity, and hence safety to gene transfer protocols by minimising ectopic transgene expression through 'transcriptional targeting'. There is additional evidence that avoiding

transgene expression in antigen presenting cells (APC's) by the use of tissue-specific promoters may be necessary and sufficient to avoid the induction of an immune response to otherwise immunogenic transgenes [Hartigan-O'Connor *et al.*, 2001; Pastore *et al.*, 1999; Weeratna *et al.*, 2001]. This may be particularly relevant for genetic diseases where the subject is naïve to the expressed transgene, and for vectors such as Ad which transduce antigen-presenting cells. Pastore *et al.* [Pastore *et al.*, 1999] demonstrated that the antibody response raised in some strains of mice against α_1 -antitrypsin expressed from an Ad could be abrogated by the use of a liver-specific promoter. Weeratna *et al.* [Weeratna *et al.*, 2001] compared the muscle-specific muscle creatine kinase (MCK) and CMV promoters in intramuscularly administered plasmid vectors to demonstrate how the immune response to a transgenic antigen (the highly immunogenic hepatitis B surface antigen) could be avoided *in vivo*. The experiment was controlled for differences in the levels of CMV and MCK induced expression, and this observation was attributed to the negligible activity of the MCK promoter in antigen-presenting cells *in vivo*.

Non Tissue-Specific Promoters

Non-specific alternatives to viral promoters have however been tested in an attempt to improve the longevity of expression *in vivo*. These include the small nuclear RNA U1b promoter [Van Linthout *et al.*, 2002], which drove expression of apoA-I expression from a first generation adenovirus *in vivo* at levels just under the apoA-I promoter, which had previously been shown to equal the strength of CMV [De Geest *et al.*, 2000]. Gill *et al.* [Gill *et al.*, 2001] evaluated the ubiquitin C (UBC) and elongation factor 1 (EF1) promoters for plasmid vector-mediated gene therapy of cystic fibrosis and were able to obtain expression for several months in the lung *in vivo*, whilst CMV promoter-induced expression had declined by two weeks. Ramezani *et al.* [Ramezani *et al.*, 2000] examined the activity of two alternative LTR promoters, and the non-specific EF1 and human phosphoglycerate kinase 1 (PGK1) promoters in a panel of retroviral vectors. Excluding CMV, the EF1 promoter was found to induce the strongest levels of transcription of several promoters tested, and it far exceeded CMV in a haematopoietic cell line. The non-specific PGK1 promoter was also found to exceed CMV in another study, driving reporter gene expression from an Ad in haematopoietic stem cells *in vitro* [Fan *et al.*, 2000].

The non-specific EF1 promoter has been shown variously to exceed and outlast CMV-mediated expression from several vectors, including Ad, AAV and plasmid vectors, *in vitro* and *in vivo* [Nakai *et al.*, 1998; Ye *et al.*, 1998]. Song *et al.* [Song *et al.*, 1998] found the EF1 promoter induced levels of hAAT expression comparable to CMV in myoblasts from an AAV vector *in vitro* (~0.8 μ g/day) that remained stable following one month in culture, whilst CMV promoter induced expression had declined to a tenth of its former value. However, intramuscular administration of these vectors revealed the CMV promoter-driven vectors gave ten-fold or higher levels of hAAT expression *in vivo*, at up to 800 μ g/ml. Both promoters induced expression that remained stable at 15 weeks. Neither of these constructs matched the levels of

hAAT obtained by a previous study using systemically delivered adenoviral vectors, which observed transient high expression from the CMV promoter (~20mg/ml) and sustained lower levels of expression from the EF1 promoter (~2mg/ml) [Guo *et al.*, 1996]. EF1 has also been utilised in a 3rd generation adenoviral system, from which it was able to sustain therapeutic levels of erythropoietin *in vivo* that were maintained one year post-infection (depending on mouse strain) [Maione *et al.*, 2000]. The PGK promoter has also been utilised in an HCAAd vector, where it was found to induce expression levels comparable to the CMV promoter, but at 100-fold less than the hAAT promoter [Schiedner *et al.*, 2002]. Finally, the housekeeping glucose 6-phosphate dehydrogenase promoter was found to reduce the variegation effect associated with retroviral vectors driven by LTR promoters, albeit at significantly lower levels *in vitro* [De Angioletti *et al.*, 2001].

Tissue-Specific Promoters

Endogenous eukaryotic promoters have typically proved inferior to viral promoters in terms of expression intensity, but it is likely that this may be overcome by improvements to current eukaryotic promoter constructs. Eukaryotic genes are highly regulated and it is to be expected that success in their use will depend upon the use of an appropriate promoter and the incorporation of additional elements to maximise its expression, in contrast to the frequently more compact, self-contained viral promoters. The list of tissue and disease-selective eukaryotic promoters employed to drive target-specific transgene expression has expanded considerably over the past few years and reflects attempts to improve the strength and longevity of transgene expression *in vivo* (see Table 1).

Liver Promoters

Whilst first-generation Ads are typically unable to sustain transgene expression *in vivo* for over 28 days, De Geest *et al.* [De Geest *et al.*, 2000] used the apolipoprotein (apo) A-I promoter in conjunction with its intron and enhancers to obtain significant levels of apo A-I (>20mg/dl; comparable to CMV-induced levels) from the liver of C57BL/6 mice using a first-generation Ad. This study was intended to evaluate the potential for overexpression of HDL in ischaemic cardiovascular disease and was able to achieve expression for up to six months in mice using the apo A-I promoter/enhancer cassette in a first generation Ad. The CMV promoter driven construct however, provoked an inflammatory response and hepatotoxicity with concomitant loss of expression by day 35 [De Geest *et al.*, 2000]. A combination of the powerful human α_1 -antitrypsin (hAAT) promoter and apo A-I enhancers successfully improved upon these levels several-fold further to >300mg/dl [Van Linthout *et al.*, 2002]. Similarly, in contrast to CMV promoter-driven HCAd, transgene expression for periods of up to a year or more have been obtained utilising eukaryotic promoters in third-generation Ad vectors. The apoE promoter has sustained high-level apoE expression for over two years from an HCAAd in a apoE^{-/-} mouse model of monogenic hyperlipidaemia, correcting hypercholesterolaemia in the mice for the duration of their natural life [Kim *et al.*, 2001]. Oka *et al.* [Oka *et al.*, 2001] were similarly able to prevent

Table 1. Transcriptional Targeting using Eukaryotic Promoters in Gene Therapy

Target tissue	Promoter	Vector	Transgene	References
LIVER	Apo A-I	Ad	Apo A-I	[De Geest <i>et al.</i> , 2000]
	ApoE	HCAd	ApoE	[Kim <i>et al.</i> , 2001]
	α_1 -antitrypsin (hAAT)	Ad HCAd Plasmid	Apo A-I hAAT factorIX	[Van Linthout <i>et al.</i> , 2002] [Schiedner <i>et al.</i> , 1998] [Schiedner <i>et al.</i> , 2002] [Miao <i>et al.</i> , 2001] [Ehrhardt <i>et al.</i> , 2002]
	hAAT & Apo A-I	Retroviral	hAAT	[Okuyama, 1996]
	Transthyretin	HCAd	hGH	[Burcin <i>et al.</i> , 1999]
	Liver-enriched activator	Transgenic	LUC	[Kistner <i>et al.</i> , 1996]
	Albumin	HCAd Lentivirus	FactorVIII factorIX	[Reddy <i>et al.</i> , 2002] [Follenzi <i>et al.</i> , 2002]
	Phosphoenolpyruvate carboxykinase (PEPCK)	HCAd	VLDLR	[Oka <i>et al.</i> , 2001]
	RNAP _{II} promoter	Retrovirus	hAAT	[Rettinger <i>et al.</i> , 1994]
ENDOTHELIUM	PAI-1	AAV	Thrombomodulin	[Mimur J, 2001]
	ICAM-2, Endoglin	Plasmid	Endoglin	[Velasco <i>et al.</i> , 2001]
	ICAM-2, flt-1, vWF	Ad	lacZ	[Nicklin <i>et al.</i> , 2001]
MUSCLE	MCK	Ad Plasmid Ad/AAV	LacZ, LUC hBSAg -sarcoglycan	[Hauser <i>et al.</i> , 2000] [Larochelle <i>et al.</i> , 2002] [Weeratna <i>et al.</i> , 2001] [Cordier <i>et al.</i> , 2000]
	SMC α -actin	Plasmid Ad Ad AAV	LUC Rb/E2F hybrid GFP, lacZ, IFN Factor IX	[Keogh <i>et al.</i> , 1999] [Prentice <i>et al.</i> , 1997] [Wills <i>et al.</i> , 2001] [Ribault <i>et al.</i> , 2001] [Hagstrom <i>et al.</i> , 2000]
	Myosin heavy-chain	Plasmid AAV	CAT lacZ, hGH	[Skarli <i>et al.</i> , 1998] [Aikawa <i>et al.</i> , 2002]
	Myosin light-chain	Ad AAV	LacZ, LUC GFP, antisense	[Griscelli <i>et al.</i> , 1998] [Franz <i>et al.</i> , 1997] [Phillips <i>et al.</i> , 2002]
EPITHELIUM	Cytokeratin 18	Plasmid	LacZ, CFTR	[Chow <i>et al.</i> , 1997] [Koehler <i>et al.</i> , 2001]
	CFTR	Ad	LacZ, LUC	[Imler <i>et al.</i> , 1996] [Suzuki <i>et al.</i> , 1996]
NEURONAL	GFAP, NSE, Synapsin I, Preproenkephalin, Dopamine - hydroxylase (d H)	Ad AAV Plasmid, Ad	LacZ, GFP LUC, GFP CAT, GFP, lacZ	[Smith-Arica <i>et al.</i> , 2000] [Glover <i>et al.</i> , 2002] [Xu <i>et al.</i> , 2001] [Hwang <i>et al.</i> , 2001]

(Table 1). Contd....

Target tissue	Promoter	Vector	Transgene	References
	Prolactin	Ad	LacZ, HSV-tk	[Southgate <i>et al.</i> , 2000]
	Myelin basic protein	AAV	GFP	[Chen <i>et al.</i> , 1998]
ERYTHROID	Ankyrin	Retrovirus Lentivirus	-globin ferrochelataase	[Sabatino <i>et al.</i> , 2001] [Richard <i>et al.</i> , 2001]
	-spectrin, Globin	Lentivirus	GFP, / -globin	[Moreau-Gaudry <i>et al.</i> , 2001]
	HLA-DR	Lentivirus	GFP	[Cui <i>et al.</i> , 2002]
	CD4	Retroviral	GFP	[Zhao-Emonet JC, 2000]
	Dectin-2	Plasmid	GFP, LUC	[Morita <i>et al.</i> , 2001]

ABBREVIATIONS: PAI-1, plasminogen activator inhibitor 1; ICAM-2, intercellular adhesion molecule2; flt-1, *fms*-like tyrosine kinase-1; vWF, von-Willebrand factor; MCK, muscle creatine kinase; CFTR cystic fibrosis transmembrane conductance regulator; GFAP, glial fibrillary acidic protein; NSE, neuronal-specific endolase; LUC, luciferase; GFP, green fluorescent protein; HSV-tk, herpes simplex virus thymidine kinase.

the onset of aortic atherosclerosis in LDLR^{-/-} mice by driving expression of very low-density lipoprotein (VLDLR) from a liver-specific phosphoenolpyruvate carboxykinase (PEPCK) promoter for at least six months from a HCAd vector.

Numerous other promoters have also been evaluated for hepatocyte-specific expression in liver disorders, such as α_1 -antitrypsin deficiency and haemophilia, which typically require stable long-term transgene expression. A haemophilia B study that made use of the hAAT promoter in 1st and 3rd generation Ad vectors was able to demonstrate stable expression of factor IX for up to 8 months at very high levels (up to 100 μ g/ml) when these vectors were systemically delivered into C57BL/6 mice [Ehrhardt *et al.*, 2002]. A gradual decline in transgene expression at post 4 months from a stable level of 15 μ g/ml was attributed to cumulative loss of the episomal Ad vector genomes over time. Interestingly, differences in expression levels between first and third generation Ad vectors were comparatively small. Wang *et al.* [Wang *et al.*, 1999] utilised a composite liver-specific promoter to drive supra-physiological levels (up to 15-20 μ g/ml) of factor IX from an AAV *in vivo* for at least 5 months in haemophilia B knockout mice, demonstrating the benefits to be derived from using a specifically optimised tissue-selective promoter cassette. The promoter consisted of the thyroid hormone-binding globulin promoter sequences, two copies of an I-macroglobulin/bikunen enhancer and an optimised 71bp leader [Ill *et al.*, 1997]. Gene therapy for haemophilia does not necessarily require transcriptional targeting of the liver however, and other tissues such as muscle may serve equally well as a source of transgene product [Cordier *et al.*, 2001; Nakai *et al.*, 1998]. This derives from the fact that diseases, such as haemophilia do not require tissue-specific transgene expression, merely the sustained production of soluble

therapeutics that may be secreted from any appropriate tissue.

Vascular Promoters

A number of vascular gene therapy strategies, including those for cardiovascular disease and aimed at angiogenic endothelium in cancerous tissue, have targeted expression to vascular endothelium using eukaryotic promoters. ICAM-2 has been found to drive very high (several-fold over CMV in some cell lines) but non-specific expression in a number of cell types [Nicklin *et al.*, 2001]. This may have been due to interference from transactivating viral sequences in the first-generation Ad utilised, as has been observed for other promoters [Rubinchik *et al.*, 2001; Steinwaerder *et al.*, 2000]. This study also demonstrated that the *fms*-like tyrosine kinase-1 (flt-1) promoter was selective for endothelial cells *in vitro* and silent in the liver *in vivo* [Nicklin *et al.*, 2001]. It was subsequently utilised in an Ad system that was transductionally targeted to pulmonary endothelium using a bispecific antibody against angiotensin-converting enzyme (ACE) [Reynolds *et al.*, 2001]. The combined effect was highly synergistic, with a 300,000 fold improvement in selectivity for pulmonary endothelium over the liver *in vivo* following systemic administration. Similar concepts are now being developed for cancer [Barnett *et al.*, 2002].

Muscle Promoters

The MCK promoter has been demonstrated to drive sustained selective transgene expression for gene therapy of Duchenne's muscular dystrophy, whose treatment requires muscle-specific transgene expression [Hauser *et al.*, 2000; Larochelle *et al.*, 2002]. In skeletal muscle tissue *in vivo* Ad-mediated MCK promoter-driven expression outlasted CMV by several months. A truncated form has been used to drive

high level stable expression of α -sarcoglycan in myofibers for over 16 weeks from a first generation Ad, and for up to a year from a recombinant AAV after direct muscle injection of vector in a mouse model of muscular dystrophy; the effect was drastic reduction of fiber degeneration and fibrosis in skeletal muscle [Cordier *et al.*, 2001]. The smooth muscle cell α -actin promoter has been successfully used by several groups to drive muscle-specific expression of transgenes [Keogh *et al.*, 1999; Wills *et al.*, 2001], and Ribault *et al.* [Ribault *et al.*, 2001] were able to improve upon the levels of smooth muscle cell-specific expression by coupling either myosin heavy chain or creatine kinase enhancers to the α -actin promoter.

CNS Promoters

Several diseases similarly require expression restricted to the central nervous system. Smith-Arica *et al.* [Smith-Arica *et al.*, 2000] utilised the astrocyte-specific glial fibrillary acidic protein (GFAP) and neuronal-specific endolase (NSE) promoters to generate inducible neuro-specific vectors, and demonstrated selective expression *in vivo*, though expression levels *in vitro* were much lower than those from a non-specific α -actin/CMV hybrid promoter. Xu *et al.* [Xu *et al.*, 2001] however found several brain-specific promoters to be superior to CMV in a panel of neuron-specific AAV vectors *in vitro* and *in vivo*, including the NSE, GFAP, preproenkephalin (PPE) and non-specific elongation factor EF1 promoters. In primary cell cultures *in vitro* the EF1 and NSE promoters were at least 10 times the strength of CMV, whilst the GFAP and PPE promoters induced a comparable level of reporter gene expression. Following *in vivo* stereotactic injection of these AAV vectors, the NSE promoter was found to induce almost 100 fold greater levels of expression than the CMV promoter, whilst the EF1, GFAP and PPE promoters gave up to several-fold greater levels of expression. Addition of the woodchuck posttranscriptional regulatory element to the NSE and PPE constructs improved expression levels by up to a further 10 fold *in vitro* and *in vivo*.

Haematopoietic-Lineage Promoters

The propensity of retroviral vectors utilising LTR promoters to be silenced *in vivo* when there is no selective advantage conferred upon transduced cells has also led to a search for alternative promoters. Sabatino *et al.* [Sabatino *et al.*, 2001] utilised the human ankyrin (ANK-1) promoter to drive erythroid-specific expression of α -globin. They obtained low-level expression *in vivo* in anaemic mice following *ex vivo* transduction of haemopoietic stem cells, at approximately 8% of control mRNA levels in red blood cells at 16 weeks post-transplant. Importantly however, the ANK-1 promoter did confer position-independent and copy number-dependent α -globin expression. Moreover, the vector did not suffer from the rearrangement effects associated with the incorporation of locus control region (LCR) elements in some other globin retroviral vectors. Richard *et al.* [Richard *et al.*, 2001] used an ANK-1/ α -globin HS-40 hybrid promoter in a SIN lentiviral vector to drive expression of ferrochelatase in erythropoietic protoporphyria (EPP) (ferrochelatase deficient) mice with more success. In erythroid cells *in vitro*, this promoter induced levels that were comparable to or exceeded those driven by a CMV

promoter. *In vivo* they were able to achieve therapeutic correction in secondary-recipient EPP mice that received a (secondary) transplant of transduced haemopoietic stem cells, attaining 53% of normal ferrochelatase activity in bone marrow cells at 8 months after initial transduction. Mice were initially transplanted with *ex vivo* transduced bone marrow stem cells (primary recipient) and four months later used as donors to transplant a second batch of mice (secondary recipients) that were examined post-four months. The high efficiency of lentiviral transduction meant that the authors were able to avoid the need for sorting and preselection of transduced cells *in vitro* prior to transplantation (and thus the use of an immunogenic reporter gene). A recent paper reported the use of the α -globin promoter and associated LCR elements in a lentiviral vector to express a reporter gene at high levels in haemopoietic stem cells, which exceeded those induced by an ANK-1/ α -globin HS-40 promoter construct *in vitro* [Hanawa, 2002].

PROMOTERS FOR CANCER GENE THERAPY

The development of tissue-specific and engineered promoters has been most intense in the field of cancer gene therapy, despite the fact that a viral promoter might be considered ideal in the context of achieving the high-levels of transient expression required for a cytotoxic or immunostimulatory effect (reviewed in [Harrington *et al.*, 2002; Nettelbeck *et al.*, 2000]). However, in most applications the toxicity of the therapy necessitates the stringent targeting of vectors if a favourable therapeutic index is to be obtained. This is particularly true if vectors are ever to be delivered systemically rather than by local injection, which may not always be suitable for certain types of inaccessible or metastatic cancer. This consideration has also led to the development of highly specific conditionally-replicating or 'oncolytic' adenoviruses (CRAAs). Typically the generation of Ads competent to replicate selectively in cancer cells has been via either deleting the parts of the virus required for replication in quiescent cells, or by placing transcription of key viral proteins (e.g. E1a) under the control of promoter elements that are only active in cancer cells (or both) (reviewed in [Krutz *et al.*, 2002; Ring, 2002]). It is likely however, that this strategy will still require transductional targeting of Ads, in order to overcome the poor efficiency with which wild type Ads transduce many primary cancer cells due to lack of coxsackie adenovirus receptor (CAR) expression. For example, Barnett *et al.* [Barnett *et al.*, 2002] were able to demonstrate greatly improved selectivity of a transcriptionally and transductionally targeted adenoviral vector for osteosarcoma cells *in vitro*. Untargeted (CMV promoter-induced) vectors exhibit 15 fold higher expression in HepG2 hepatocytes over 143B osteosarcoma cells, whilst the dually targeted vector (utilising the osteocalcin promoter) had a 44 fold higher expression in 143B cells over HepG2's, and a 665 times greater transduction selectivity for 143B cells versus HepG2's compared to the untargeted vector (assayed by gene expression).

The range of cancer-specific promoters evaluated to date is enormous, and reflects the diversity of cancers that they have been designed to target (see Table 2). A powerful alternative methodology has been to target angiogenic

Table 2. Transcriptional Targeting in Cancer Gene Therapy

Promoter	Cancer type	References
-fetoprotein (AFP)	Hepatocellular Carcinoma	[Hallenbeck <i>et al.</i> , 1999] [Ishikawa <i>et al.</i> , 1999] [Kanai, 2001]
carcinoembryonic antigen (CEA)	CEA-expressing tumours	[Brand <i>et al.</i> , 1998] [Cao <i>et al.</i> , 2001] [Qiao <i>et al.</i> , 2002]
erbB2	ERBB2-expressing tumours	[Vassaux <i>et al.</i> , 1999]
mucin-1 (muc1)	MUC1 (DF3)-expressing tumours	[Stackhouse <i>et al.</i> , 1999] [Tai <i>et al.</i> , 1999]
L-plastin (LP-P)	Epithelial-derived tumours	[Chung <i>et al.</i> , 1999]
-lactalbumin (ALA),	Breast	[Anderson <i>et al.</i> , 2000]
midkine (MK)	Pancreatic	[Yoshida <i>et al.</i> , 2002]
cyclooxygenase-2 (COX-2)	Gastrointestinal	[Yamamoto <i>et al.</i> , 2001] [Wesseling <i>et al.</i> , 2001]
PSA/PMSA, kallikrein-2	Prostate	[Xie <i>et al.</i> , 2001] [Li <i>et al.</i> , 2002] [Rodriguez <i>et al.</i> , 1997] [Pan <i>et al.</i> , 1999] [Wu <i>et al.</i> , 2001]
probasin (ARR2PB)	Prostate	[Lowe <i>et al.</i> , 2001; Rubinchik <i>et al.</i> , 2001]
tyrosinase promoter	Melanoma	[Park <i>et al.</i> , 1999]
hypoxic response elements (HRE)	Not tissue-specific	[Dachs <i>et al.</i> , 2000; Ruan H, 2001]
hTERT	Not tissue-specific	[Gu <i>et al.</i> , 2000; Koga <i>et al.</i> , 2000; Lin <i>et al.</i> , 2002; Majumdar <i>et al.</i> , 2001]
flt-1, flk1/KDR, E-selectin, endoglin, ICAM-2, preproendothelin 1 (PPE-1)	Angiogenic tumour vasculature	[Jaggar <i>et al.</i> , 1997] [Walton <i>et al.</i> , 1998] [Savontaus <i>et al.</i> , 2002] [Bauerschmitz <i>et al.</i> , 2002] [Velasco <i>et al.</i> , 2001] [Varda-Bloom <i>et al.</i> , 2001]
prolactin (PRL)	Pituitary tumours	[Southgate <i>et al.</i> , 2000]
osteocalcin 2	Osteosarcoma	[Barnett <i>et al.</i> , 2002]

ABBREVIATIONS: PSA/PMSA, prostate-specific membrane antigen; hTERT, human telomerase reverse transcr

tumour vascular endothelium, which largely circumvents the problem of specifically targeting the genetic heterogeneity within and between different cancers. Angiogenesis is essential for tumour growth, invasion and metastasis and antiangiogenic therapy potently inhibits tumour growth. This has been achieved both by transductional [Hood *et al.*, 2002] and transcriptional [Savontaus *et al.*, 2002] targeting of tumour angiogenesis.

The relative expendability of prostate and melanocyte tissue has permitted the use of tissue rather than tumour specific promoters for applications targeted to these sites. The prostate-specific membrane antigen (PSA/PMSA) and kallikrein-2 promoters have been widely used to drive prostate-specific expression of cytotoxic genes [Li *et al.*, 2002; Wu *et al.*, 2001; Xie *et al.*, 2001] or generate oncolytic viruses [Rodriguez *et al.*, 1997]. Lowe *et al.* [Lowe *et al.*, 2001] improved upon the expression levels obtained from the PSA promoter by using a modified probasin (ARR2PB) promoter *in vitro* and *in vivo* in an adenoviral vector. Vectors for therapy of melanoma have mostly used the tyrosinase promoter, which is selectively expressed in melanocytes and most melanomas [Nettelbeck *et al.*, 2002; Park *et al.*, 1999].

Nettelbeck *et al.* [Nettelbeck *et al.*, 2002] recently generated a melanoma targeted oncolytic CRAd vector by placing expression of an E1a mutant transgene under the control of a tyrosinase promoter. Wild-type Ads use E1a to sequester pRb and p300 and so induce the cell to start DNA synthesis; DNA viruses such as adenoviruses infect quiescent cells and induce them into the S phase of the cell cycle so that viral DNA replication can proceed. The E1a protein of human adenoviruses, which binds pRb, p300, and other transcription factors, is largely responsible for this forced entry into the S phase. The mutant E1a protein expressed from this vector is unable to bind either pRb or p300 (hence the virus is inactive in normal quiescent cells), but is still able to perform its other functions of viral and cellular gene transactivation. Thus this vector replicates at wild-type efficiency in cancer cells, but with up to 1000-fold attenuated cytotoxicity in control (non-melanoma) cells. Critically, only small amounts of E1a are required for viral replication and so specific but (relatively) weak eukaryotic promoters are not a drawback to the functional efficiency of such CRAd vectors.

Perhaps the most obvious promoter candidates for use in generating vectors with targeted activity are the deregulated cell cycle genes that are definitive of most types of cancer, such as the tumour suppressors p53 and retinoblastoma (Rb) protein. Deletion or mutation of the p53 tumour suppressor gene occurs frequently (>50%) in most types of human cancer and tumours lacking functional p53 are in many cases refractory to chemotherapy or radiation. The E1B viral gene encodes a 55-kD protein that binds and inactivates p53, and prevents p53 induced-apoptosis in transduced cells. Deleting the E1B-55K adenoviral gene permits the selective replication of these viruses in cells lacking functional p53 [Bischoff *et al.*, 1996]. A p53-dependent Ad expression cassette was similarly effective [Lipinski *et al.*, 2001]. This latter consisted of two components; a lac repressor (lacI) inducible by wild-type p53, and a therapeutic transgene under control of the heat shock 70 (hsp70) promoter incorporating LacI binding sites. In cancerous cells devoid of functional p53 there is no induction of the lac repressor and the transgene is expressed from the hsp70 promoter, which is active in most tumour cells.

The retinoblastoma/E2F signalling pathway is deregulated in almost all tumour cells and has also been exploited by groups seeking to generate selective oncolytic viruses. Johnson *et al.* [Johnson, 2002] achieved this by making a deletion in the adenoviral E1A region and replacing the viral E1A and E4 promoters with the human E2F1 promoter, which is directly repressed by pRB, to create a CRAd selective for cells with defects in the pRB pathway. Cytolytic effects on tumour lines *in vitro* were comparable to wild-type virus and reduced by 100-1000 fold in normal cells. *In vivo*, the virus exhibited reduced toxicity and conferred a survival advantage on cancer xenograft mice.

Ramachandra *et al.* [Ramachandra *et al.*, 2001] generated an oncolytic vector with improved specificity by also deleting a portion of E1a, and additionally engineering the virus to express a potent E2F antagonist from a p53-inducible promoter. Thus, in cells functionally expressing p53, the antagonist is induced and the cellular and viral transactivating functions of E2F required for viral replication are blocked. A copy of the adenoviral major late promoter was also introduced into the E3 region to overexpress E3-11.6K (an adenoviral protein involved in cell lysis) to enhance the cytolytic effect of the virus (this function does not become activated until the transactivating functions of E2F have taken effect). Over 50% of animals with intraperitoneal tumours treated with this virus survived and were tumour-free by 100 days. Tumour xenograft growth could also be efficiently inhibited (43%-75% depending on the model), and the virus was found to be up to 1,000 fold more potent than an E1B-55K deleted virus.

REGULABLE PROMOTERS

Ligand-Inducible Systems

The majority of regulable systems engineered to date are ligand inducible promoters, which have the distinct advantage of permitting pharmacological control of transgene expression following vector administration *in vivo* (reviewed in [Zoltick *et al.*, 2001]). Such systems also permit the production of viruses encoding toxic transgenes whose

constitutive expression would otherwise kill the packaging cell line. In some scenarios, such as the administration of vectors encoding proteins with a narrow therapeutic index, or where expression is required only transiently at a specific time, such regulation may be essential.

The most prominent regulable system has been the tetracycline (tet) on/off system, which is based on the highly-sensitive prokaryotic tetracycline resistance operon and has demonstrated a highly suitable kinetic and dose-response profile in numerous studies *in vitro* and *in vivo* [Blau *et al.*, 1999; Gossen *et al.*, 1995; Rossi *et al.*, 1998; Urlinger *et al.*, 2000] (see Fig. 1). In this system, the transactivator (TA) is constitutively expressed (or optionally via a tissue-specific promoter), whilst the transgene is under control of the tet-response element (tet operator). The TA consists of the tet repressor fused to a suitable transcription factor activation domain. The transactivator and transgene in this system may be encoded by the same or two separate vectors, but the latter 'dual' vector system has had more success in maintaining regulable stringency. The original wild-type repressor requires constant administration of the drug to keep the transactivator (tTA) displaced from the response element and the switch 'off' (tet-OFF system). The most useful tet switches for many gene therapy applications are likely to be those that utilise the mutant 'reverse' tet repressor (rtTA), which binds in the presence of tetracycline (or a suitable analogue). This switch is constitutively 'off' and permits the 'on' signal to be transmitted upon administration of tetracycline (the tet-ON system), necessitating its administration only when transgene expression is required rather than vice versa. A very large number of studies have made use of this system, which permits regulation of transgene expression by simple oral administration of doxycycline; recent examples of gene therapy applications of the tet switch include regulated *in vivo* expression of leptin [Wilsey *et al.*, 2002], erythropoietin [Rendahl *et al.*, 2002; Sommer *et al.*, 2002] and IL-10 [Apparailly *et al.*, 2002].

Whilst some limitations remain, particularly regarding the stringency of basal expression and optimisation of tet-ON inducibility [Mizuguchi *et al.*, 2002; Mizuguchi H, 2001], improvements to this system are ongoing [Fitzsimons *et al.*, 2001; Lamartina *et al.*, 2002; Vigna *et al.*, 2002]. One recent attempt to improve the tet-OFF system fused the tet repressor to mSin3, which is involved in transcriptional repression by histone deacetylation, and reduced the levels of expression in the 'off' state by up to 25-fold [Jiang *et al.*, 2001].

Steroid response switches using non-human analogues have also proved successful because of their suitable pharmacokinetic properties as small fast-acting and rapidly metabolised lipophilic molecules [Hoppe *et al.*, 2000; Suhr *et al.*, 1998]. Whilst their use *in vivo* might ultimately prove problematic because of the physiological side-effects of steroid ligands, the extreme affinity of mifepristone (RU486) for the mutated progesterone nuclear receptor at least, has facilitated the development of a responsive switch that functions at levels of hormone that have no endogenous activity [Burcin *et al.*, 1999; Pierson *et al.*, 2000; Wang *et al.*, 1997]. This system utilises a modular recombinant receptor consisting of the mutated ligand-binding domain

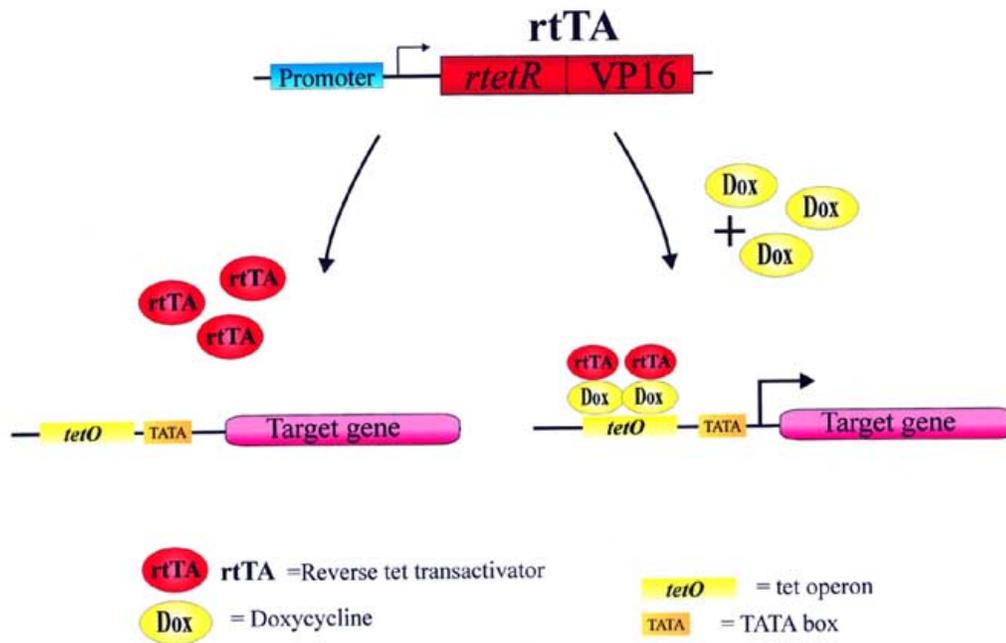


Fig. (1). The tet-ON switch consists of two components; the reverse tet transactivator, which may be transcribed from a constitutive or tissue-specific promoter, and the tet-responsive *tetO* operon, which directs expression of the target gene. In the presence of a tet analogue, such as doxycycline, the rtTA may bind the operon and activate transcription via its activation domain, the herpes simplex virus transcription factor activation domain VP16.

fused to appropriate transcription factor DNA-binding (such as yeast GAL4) and activation (such as herpes simplex virus VP16) domains (see 'transcription factors' for a description of these). The receptor is constitutively expressed and activates transcription from the target gene when ligand is present and binds to it. One study that evaluated the tet-ON and anti-progestin switches in a dual adenoviral vector system *in vitro* demonstrated up to 1,800 and 600 fold induction levels with the two switches, respectively [Molin *et al.*, 1998]. Xu *et al.* [Xu *et al.*, 2001] used the ligand binding domain of the oestrogen receptor fused to a zinc finger DNA-binding domain and demonstrated over 500-fold induction upon administration of tamoxifen using this ligand switch in an adenoviral system *in vitro*.

Another study utilising an ecdysone insect hormone response switch demonstrated superior induction response profiles to a tet switch system, with lower basal activity and higher inducibility [No *et al.*, 1996; Saez *et al.*, 2000]. As with the mifepristone switch, this system benefits from the potent transcriptional activating capabilities of nuclear hormone receptors [Collingwood *et al.*, 1999; Glass *et al.*, 2000]. It consists of an engineered ligand binding domain fused to DNA-binding and activation domains; upon ecdysteroid ligand binding (or the ecdysone analogue muristerone A), the receptor heterodimerises with the retinoid X receptor (RXR) and activates transcription from genes bearing the target DNA sequence. Saez *et al.* [Saez *et al.*, 2000] recently identified ponasterone A as an alternative phytoecdysteroid inducer derived from plants, that is inert in mammals and ~1000 fold more active than 20 OH-ecdysone. When used in conjunction with RXR ligands these

synergised to dramatically improve absolute levels of induction by up to five fold.

A third strategy has utilised the dimerising function of the antibiotic rapamycin, which links the two proteins FK506-binding-protein (FKBP) and FKBP12-rapamycin-associated protein (FRAP). This promoter construct utilised two synthetic transcription factors, one containing a DNA-binding domain fused to FKBP and another the activation domain of NF- κ B p65 fused to FRAP. The promoter containing the target DNA sequence is only induced when the two are dimerised by the action of rapamycin, though the adverse physiological properties of rapamycin have necessitated the use of alternative analogues *in vivo* [Rivera *et al.*, 1999; Ye *et al.*, 1999]. Ye *et al.* [Ye *et al.*, 1999] utilised two AAV vectors, one expressing the dimerising transcription factors and the other an erythropoietin transgene under transcriptional control of the target promoter. When delivered at a 1:1 ratio by intramuscular injection in mice, plasma levels of erythropoietin were inducible by up to 200-fold (up to 700 mU/ml) upon administration of rapamycin in a dose-responsive fashion at six months after injection. Administration of vectors to non-human primates permitted 50-fold induction of erythropoietin from a baseline of 2mU/ml to up to 100mU/ml that returned to baseline in 14 days. This maximal level of induction was comparable to the levels of erythropoietin expressed from a CMV-promoter driven vector. This inducibility was however, diminished 20-fold by three months and was negligible by four months; a loss tentatively attributed to the immunogenicity of the viral domains incorporated into the synthetic transcription factors.

It may be observed that all of these systems have taken advantage of the modular nature of transcription factors to combine different DNA-binding and activation domains in recombinant transcription factors to facilitate the engineering of selective inducible promoter systems. Whilst many early systems have utilised highly active activation domains, such as that derived from the herpes simplex VP16 factor, it is possible that the immunogenicity and promiscuous activity of these will render them less desirable in future systems [Ye *et al.*, 1999]. Similarly, the use of designed novel zinc-finger DNA-binding domains to regulate expression from endogenous or introduced transgenes is likely to be more favourable than the use of non-human subunits, such as the yeast GAL4 DNA-binding domain. A recent report highlighted the difficulties that may be associated with the use of antigenic components such as the rtTA *in vivo*, though results in this study are likely to have been influenced by the immunogenicity of the plasmid and first generation Ad vectors used (both of which were driven by viral promoters) [Latta-Mahieu *et al.*, 2002].

Stimulus-Inducible Systems

Other types of regulable promoters are those incorporating elements that are stimuli responsive, such as those inducible by heat [Lee *et al.*, 2001], radiation [Marples *et al.*, 2000], or metal-responsive elements (MREs) [Steinwaerder *et al.*, 2000]. In the last study Steinwaerder *et al.* [Steinwaerder *et al.*, 2000] utilised an insulator element to shield their regulatory cassette from transactivating interference from Ad vector sequences (that are retained in all Ad vectors). When coupled to a core promoter (consisting of a TATA box and tet operator) the level of induced expression using an MRE in this vector was comparable to that obtained from a RSV promoter. The insulating elements permitted basal (non-induced) expression to be reduced to barely detectable levels, whilst oral administration of ZnSO₄ could induce transgene expression by factors of 40 and 230-fold *in vitro* and *in vivo*, respectively.

Of significant interest to several ischaemic and cancer disease therapies are hypoxic response elements (HRE) that are activated in cells via hypoxia-inducible factor-1 (HIF-1) and permit the selective induction of gene expression in an hypoxic environment. Many groups have utilised HREs to generate synthetic hypoxia-inducible promoter constructs. Houston *et al.* [Houston *et al.*, 1999] similarly developed shear-stress inducible promoter cassettes for application in cardiovascular gene therapy by the incorporation of shear-stress response elements (see 'rational design').

Depending on the disease context, it may be necessary to generate physiologically responsive self-regulating constructs that adjust their transgene expression levels, such as those aimed at treating diabetes. Lee *et al.* [Lee *et al.*, 2000] generated an auto-regulating insulin expressing AAV for therapy of type I diabetes by utilising the hepatocyte-specific L-pyruvate kinase (LPK) promoter, which responds to blood glucose levels and observed remission of diabetes in streptozotocin-induced diabetic rats and autoimmune diabetic mice. Another group engineered the glucose-responsive elements from this promoter into the hepatocyte-specific insulin-like growth factor binding protein-1 (IGFBP-

1) promoter, which is negatively regulated by insulin levels [Thule *et al.*, 2000]. Chen *et al.* [Chen *et al.*, 2001] used the glucose-6-phosphate (G6Pase) promoter, which is both inducible by glucose and suppressible by insulin, in a first-generation Ad. This transcriptionally auto-regulated vector was able to achieve physiologically regulated insulin expression *in vivo* in streptozotocin-induced (type 1) diabetic nude rats.

RATIONAL DESIGN DE NOVO

Whilst the precise mechanisms of gene expression are still in the process of being fully elucidated, significant progress has been made in designing synthetic promoters. An understanding of the role of core promoters and transcription factors in effecting eukaryotic transcription is particularly relevant to many aspects of synthetic promoter construct design.

Core Promoter Structure

Control of eukaryotic gene expression is primarily effected through the activity of RNA polymerase II (RNAP_{II}) and the basal transcription machinery that is recruited to the core promoter elements found in every human gene (see [Orphanides *et al.*, 2002] for an up to date review). The RNAP_{II} and its basal transcription factors (TF_{II}s) are assembled upon several core promoter elements spanning the region -35 to +35, though it is likely that those identified to date do not constitute all of the core elements involved in promoter recognition [Roeder, 1996; Weinzierl, 1999] (Fig. 2).

The TATA box (consensus T A T A A/T A) is usually found at -25 but notably absent from the promoters of many housekeeping genes [Smale, 1997]. The initiator (Inr) (generally rich in pyrimidines), is centred on the transcription start site at -3 to +5, and is capable of supporting transcription in the absence of a TATA box, or synergising with it when both are present [Martinez *et al.*, 1995]. The downstream promoter element (DPE) frequently appears to act as an alternative to the TATA box [Zhou *et al.*, 2001] and is located around +30 (typically +28 to +34) [Burke *et al.*, 1997;1996], whilst the TFIIB recognition element (BRE) is a G C rich box that lies immediately upstream of the TATA box [Littlefield *et al.*, 1999].

The many basal factors involved in transcription are required by the RNAP_{II} to both locate the promoter via these motifs and assemble a transcription competent 'pre-initiation complex' (PIC), (consisting of the RNAP_{II} and basal factors) upon it. In addition to their intrinsic diversity, there is surprisingly variable requirement for the TATA, Inr and DPE core elements in eukaryotic promoters, and some promoters lack any of these elements [Smale, 2001]. It is known that different core promoter elements can alter promoter activation response profiles [Chalkley *et al.*, 1999], and the differential incorporation of these core motifs may increase the scope for combinatorial regulation of genes, by permitting selective communication between these elements and different enhancers, and the proteins that bind them [Butler *et al.*, 2001]. It has also become clear that the use of tissue-specific basal components further increases the potential complexity of combinatorial transcription factor-

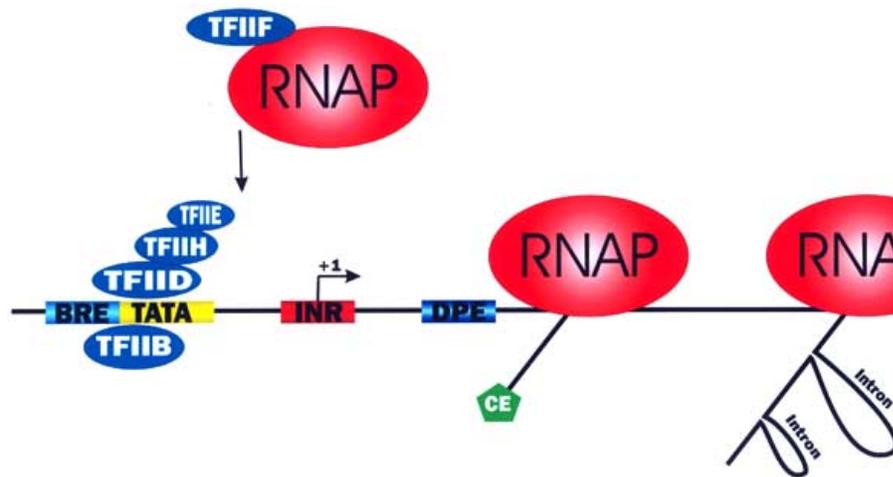


Fig. (2). A simplified schematic of transcription initiation and elongation; this diagram omits numerous components, including factors implicated in the holoenzyme model, and the array of factors that are engaged by the elongating RNAP and carry out co-transcriptional RNA processing (capping, splicing, polyadenylation): see text for details. The RNA capping enzyme ('CE') is included. RNAP = RNA polymerase, TFII = RNAP_{II} basal transcription factors.

basal factor interactions [Holmes *et al.*, 2000; Rabenstein *et al.*, 1999; Yamit-Hezi *et al.*, 1998].

Transcription Factors

The basal machinery is necessary and sufficient to generate basal levels of transcription *in vitro*, but a far more sophisticated level of control is introduced by its interaction with sets of additional *cis*-acting sequence motifs. These are recognised by gene, stimuli or tissue-specific transcription factors that act as activators or repressors of transcription, or indeed both, depending on the context.

Transcription factors commonly manipulate the activity of the basal machinery, for example by influencing recruitment of basal factors or assembly of the PIC, typically via co-regulators, which act as mediators between basal factors and transcription factors and often possess enzymatic functions (reviewed in [Glass *et al.*, 2000; Lemon *et al.*, 2000]). They may also act to regulate gene expression by assisting the processivity of the RNAP_{II} through 'pause' or 'arrest' sites, and the elongation competence of the RNAP_{II}. It is their coordinate regulation and interaction that provides the primary means by which cells control the levels of specific gene expression. It is also the means by which cells are able to effect complex global networks of transcription, and orchestrate gene expression programmes in response to various stimuli and at various stages of development (reviewed in [Lemon *et al.*, 2000]). The short *cis*-acting motifs (typically <10bp) that recruit these factors are thus, of considerable significance in the choice and design of promoters for use in targeting and controlling transgene expression, and are one of the key means by which expression may be tailored to a specific target or response. Transcription factor binding sites may lie either up or downstream of the transcription start site, and at up to 10's of kb from the core promoter, with transcription factors being brought into proximity with the promoter by 'looping' of the intervening DNA during activation [Merika *et al.*, 2001].

Nearly all transcription factors possess defined modular DNA-binding domains via which they contact these sequence motifs; there are a few that do not directly bind DNA, such as the adenoviral E1a protein and these 'hijack' the activity of others that can bind [Liu *et al.*, 1994]. In addition, they possess modular activation domains (and often several of them) that make the protein-protein contacts necessary for their function following DNA-binding, such as contacting components of the basal machinery and coactivators [Green, 2000]. These activation domains are also responsible for the interactions between transcription factors that result in their coordinate or synergistic binding properties at enhancer motifs, which makes use of the corresponding binding sites particularly advantageous [Carey, 1998; Weinzierl, 1999]. Multiple activation domains on a factor may act independently or cooperatively, and the binding of these factors on the DNA template may induce allosteric changes in these domains that are necessary for their function [Lefstin *et al.*, 1998].

Transcription Factor Design

The modular nature of transcription factor DNA-binding and activation domains lends itself to their use as 'building blocks' in the construction of novel transcription factors that may be used to drive expression from synthetic or endogenous promoters. This property has been extensively exploited in the generation of inducible 'gene-switches', and in the manufacture of entirely novel transcription factors able to bind any defined DNA sequence [Beerli *et al.*, 2002; Dreier *et al.*, 2001; Moore *et al.*, 2001; Yaghami *et al.*, 2002; Zhang *et al.*, 2000]; using engineered transcription factors to control gene expression may prove a very powerful strategy in the future of gene therapy.

Beerli *et al.* [Beerli *et al.*, 2000] designed synthetic zinc finger based transcription factors that were able to bind selectively to the promoters of the *erbB2* or *erbB3* genes and up or downregulate gene expression by several-fold, depending upon whether the zinc fingers were fused to the

VP64 viral activator or eukaryotic KRAB repressor domain. This study demonstrated the feasibility of utilising a single dominantly-acting transcription factor to control expression of a gene delivered by a retroviral vector *in vitro*, and may have significant utility in a gene therapy system.

Others seeking to design cancer-specific promoters have generated synthetic transcription factors that incorporate tissue and cell-cycle specific regulation in their design [Jerome *et al.*, 1998; Nettelbeck *et al.*, 1999]. Nettelbeck *et al.* [Nettelbeck *et al.*, 1999] generated a chimeric transcription factor consisting of the yeast GAL4 DNA-binding domain and NF-Y transactivation domain, tissue-specifically expressed from a tyrosinase promoter (consisting of two copies of the tyrosinase distal enhancer and a core tyrosinase promoter). The target sequence upstream of the reporter gene consists of multimerised GAL4 binding sites and a CDE/CHR sequence module. In quiescent cells this module is occupied by the transcriptional repressor CDF-1, and transactivation by NF-Y is blocked; in proliferating cells the CDF-1 is displaced and NF-Y is able to transactivate expression of the reporter gene. *In vitro*, plasmid constructs expressed reporter gene with cell-type specificity of 58-73-fold and cell cycle regulation of 23-fold, and expression was undetectable in non-proliferating cells and non-target cells.

A modification of the ligand-inducible systems are 'two-step transcription amplification' (TSTA) systems. These similarly utilise a synthetic inducible transactivating transcription factor to regulate expression of transgene under control of a second target promoter. However, in this case the regulating transcription factor is not ligand inducible, but constitutively active and expressed from a tissue (or disease) specific promoter. The advantage of incorporating this extra step is that when a transactivator, such as a GAL4-VP16 hybrid is used, expression is often amplified several fold over that obtained if the transgene is placed under direct control of the tissue-specific promoter. This system has been employed to improve upon prostate-specific androgen-inducible transgene expression for cancer imaging [Wu *et al.*, 2001; Zhang *et al.*, 2002], and to boost expression from a CEA promoter driven Ad [Koch *et al.*, 2001; Qiao *et al.*, 2002]. Qiao *et al.* [Qiao *et al.*, 2002] demonstrated efficacy comparable to an RSV promoter-driven vector *in vitro* and *in vivo*, but with reduced toxicity following intravenous administration. Ligand-inducible systems also benefit from this TSTA phenomenon. For example, Rubinchik *et al.* [Rubinchik *et al.*, 2001] generated a prostate-specific tet switch by placing the tet transactivator under control of the ARR2PB promoter and transgene under the tet-regulable promoter. This vector demonstrated superior expression of FasL transgene in the induced state compared to a vector with the transgene under direct control of the ARR2PB promoter. Whilst it was not as specific for prostate cells as the latter, (reflecting some persisting limitations of the tet switch), it was still specific enough for use *in vivo*. The long-term utility of these viral transcription factor domains is however uncertain.

Rational Design of Promoters

Comprehensive understanding of transcriptional mechanisms is not a prerequisite to the generation of *de novo*

synthetic promoters. Functional screening of promoter constructs can be used to isolate optimal combinations of different elements that contain the desired activity, which may be generated randomly or by incorporation of different (or multimerised) functional elements into a composite promoter. This area of research holds enormous potential for the construction of ideal compact tissue-specific promoters for any vector system.

The former approach was used by Li *et al.* [Li *et al.*, 1999], who randomly incorporated a selection of common myogenic transcription factor binding sites (E-box, MEF-2, TEF-1, and SRE sites) into a recombinant promoter library and screened several hundred to identify those with optimal muscle-specific activity. Several artificial promoters were isolated whose transcriptional potencies greatly exceeded those of some natural myogenic and viral gene promoters. Houston *et al.* [Houston *et al.*, 1999] used a similar but much smaller-scale approach to construct synthetic shear stress-inducible promoters for cardiovascular applications, evaluating only thirteen plasmid constructs. In this study they evaluated the combination and spacing of a group of several common transcription binding sites, including Sp1, NF- κ B and Ap1, in conjunction with a shear-stress response element (SSRE) to generate an inducible promoter construct that functioned from a plasmid vector delivered locally *in vivo*. Shear stress exerted on the vessel wall *in vivo* is dependent on the nature of intravascular blood flow, and expression constructs incorporating SSREs would have particular utility in occlusive vascular disease by regulating the local release of transgene product according to the presence and severity of stenosis.

Another report used high-throughput selection of synthetic promoter constructs to identify over a hundred sequences with improved activity over a minimal promoter in a neuroblastoma cell line [Edelman *et al.*, 2000]. A library of random 18mers was inserted upstream of a minimal TATA/initiator promoter and engineered into retroviral vectors and screened for activity in cells *in vitro*. The most active of those identified were screened against the TRANSFAC database (<http://transfac.gbf.de/TRANSFAC/>) and found to be a composite of several known motifs, including AP2, CEBP, GRE, Ebox, ETS, CREB, AP1 and Sp1 sites.

Other studies have conjugated different promoter elements as a simpler means of deriving synthetic promoter constructs. A composite promoter consisting of the CMV immediate early enhancer and the chicken β -actin promoter (CB) has proved to be a popular and very effective (non-specific) alternative to the CMV promoter, with superior expression profiles *in vitro* and *in vivo* (e.g. [Ramezani *et al.*, 2000; Smith-Arica *et al.*, 2000; Song *et al.*, 2001]). Xu *et al.* [Xu *et al.*, 2001] found the CB promoter to induce levels of hAAT expression that were almost 10-fold over an EF1 promoter and 137-fold over CMV-induced levels from an AAV vector administered systemically to C57BL/6 mice, attaining expression of $\sim 1.7\mu\text{g/ml}$ that was stable for at least 10 months. When used to drive expression of factor X from a AAV vectors in neonatal C57BL/6 mice, the CB promoter resulted in expression of $\sim 0.55\mu\text{g/ml}$ and $\sim 0.25\mu\text{g/ml}$ factor X from systemically and intramuscularly administered

vectors, respectively. Both remained stable for at least 15 months; normal levels are 8µg/ml and the 10% mark (0.8µg/ml) that this study approached would be therapeutic *in vivo*. Yew *et al.* [Yew *et al.*, 2001] utilised a composite ubiquitin B (UBB) promoter to induce reporter gene expression from plasmid vectors administered systemically. When coupled to a portion of the CMV enhancer the UBB promoter yielded sustained transgene expression at levels that matched those induced by the CMV promoter, and remained steady at 84 days, whilst CMV-driven expression fell to background by day 60.

Hypoxic and other Response Elements

Many studies have generated regulable promoter constructs by the incorporation of multimerised elements, such as hypoxic response elements (HRE), into composite promoters. Prentice *et al.* [Prentice *et al.*, 1997] successfully coupled these to a smooth-muscle cell specific α -actin promoter to obtain an inducible expression cassette in a plasmid vector. Others have used HREs to develop inducible viral and plasmid vectors for the treatment of ischaemic (cardiovascular and neuronal) disease or cancer, and have commonly been able to attain induced levels of expression that are comparable to those driven by non-specific viral promoters such as CMV [Binley *et al.*, 1999; Cao *et al.*, 2001; Phillips *et al.*, 2002; Post *et al.*, 2001]. Specific regulable promoters for cancer gene therapy have been generated by incorporating HREs to restrict expression to hypoxic tumour environments (reviewed in [Dachs *et al.*, 2000; Ruan H, 2001]). These have also commonly used multimerised HRE's upstream of a core promoter to drive transgene expression, inducing selective expression of the gene under hypoxic conditions. Greco *et al.* [Greco *et al.*, 2002] recently developed a chimeric promoter incorporating HRE and radiation-responsive CARG elements (derived from the Egr1 promoter) to respond to hypoxia and ionising radiation. These elements were combined and multimerised upstream of a basal CMV promoter in plasmid vectors and evaluated for their ability to respond to hypoxia and radiation stimuli in cells *in vitro*. Cells transfected with a horseradish peroxidase (HRP) expressing construct were rendered sensitive to the cytotoxic effects of prodrug (indole-3 acetic acid) administration under conditions of hypoxia and/or radiation only; in combination the cell-killing effects exceeded those of an HRP-expressing plasmid driven non-specifically by the full CMV promoter. Wu *et al.* [Wu *et al.*, 2001] were able to improve the expression levels obtained from a prostate-specific promoter without compromising its specificity by multimerising androgen-response elements (ARE's) in the PSA promoter to generate an improved androgen-inducible version for cancer gene therapy. Other elements, such as CRE's (cAMP response elements) and GRE's (glucocorticoid response elements) have also been coupled to core promoters to successfully generate powerful inducible promoter systems [Lee *et al.*, 1999; Narumi *et al.*, 1998]. Suzuki *et al.* [Suzuki *et al.*, 1996] were able to develop an inducible promoter based on the CFTR promoter, by modifying it through the addition of tandem cAMP response-elements (CRE) elements that rendered the promoter cAMP responsive *in vitro* and *in vivo*. From a low basal level, expression could be induced to up to 50% of an

RSV-LTR control by maximal upregulation of cAMP *in vitro*. When this cassette was transferred into a 1st generation Ad and administered intranasally to C57BL/6 mice, they were able to demonstrate an 11-fold upregulation of basal promoter activity in the airway epithelium using a phosphodiesterase inhibitor and cAMP analogue.

CASSETTE OPTIMISATION

The isolation or generation of an appropriate promoter is only one step in the process of optimising expression cassettes to improve transgene expression *in vivo*. The key role of chromatin structure in gene regulation suggests that elements, such as specific locus control regions (LCRs) and matrix attachment regions (MARs) should be included in gene therapy vector constructs. Whilst the ability to include specific genetic elements will depend upon the particular vector, the advent of vectors with larger packaging capacities, such as HCAAd vectors, will enable the incorporation of gene cassettes that are more homologous to naturally occurring genes, and therefore enable greater and more controlled stable gene expression.

Promoter Architecture & Chromatin Structure *In Vivo*

A large proportion of the eukaryotic genome is packaged into dense higher-order chromatin structure (heterochromatin) that renders gene promoters inaccessible to the transcriptional machinery. The regulation of gene expression in eukaryotes is thus, closely and necessarily coupled to the machinery that relieves this repression of transcription by nucleosome remodelling and reorganisation of higher order chromatin structure (reviewed in [Gasser, 2001; Narlikar *et al.*, 2002; Wolffe *et al.*, 2000]) (see Fig. 3). Any transgene cassette introduced into the nucleus must avoid the fate of being silenced by being packaged into heterochromatin, a phenomenon known as positional effect variegation (PEV) and typically observed in transduction by integrating vectors, such as retroviruses [Challita *et al.*, 1994; Rivella *et al.*, 1998]. Consequently, it is highly advantageous to incorporate elements into an expression cassette that will, at the very least, favour the retention of the cassette in a euchromatic (actively transcribed) environment, and at best, mimic the action of 'housekeeping' promoters that are maintained in a permanently nucleosome-free configuration to facilitate uninhibited transcription.

Nucleosomes and Histones

The winding of DNA round nucleosomes commonly renders regulatory motifs unrecognisable to the DNA-binding domains of transcription factors in addition to physically obstructing their access, and hence the remodelling of nucleosomes becomes necessary to permit transcription to occur [Kingston *et al.*, 1999; Tyler *et al.*, 1999]. The stability of the ionic histone-DNA interactions means transcription factors are unable to displace these structures without recruiting the assistance of specific chromatin remodelling complexes that either shift or remove nucleosomes entirely, and so 'loosen' the chromatin packaging and help the transcription machinery to navigate through it. Examples of these include the SWI/SNF and ISWI (e.g. NURF, CHRAC) ATPase chromatin remodelling

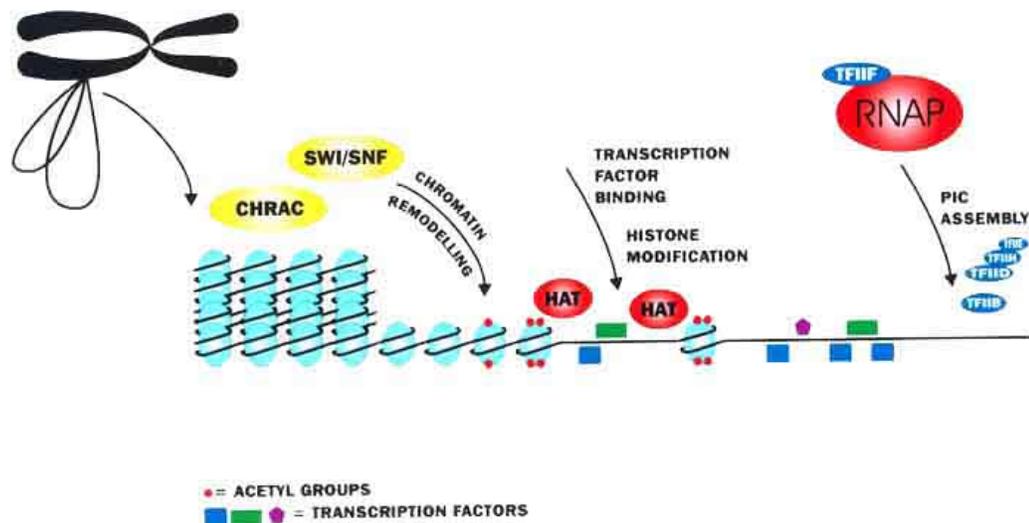


Fig. (3). A schematic representation of the processes of chromatin remodelling, nucleosome modification and transcription factor binding: these may occur in different orders to mediate PIC recruitment and transcriptional activation of a gene (see text for details).

complex families. Closely associated with this regulation by active chromatin remodelling is the manipulation of histone function by post-translational modification, (the 'histone code') [Berger, 2001; Jenuwein *et al.*, 2001; Strahl *et al.*, 2000]. Acetylation of histone N-terminal tails is now known to be particularly important, and another major class of chromatin modifying complexes is known to utilise this function [Brown *et al.*, 2000; Eberharter *et al.*, 2002]. In the majority of cases hyperacetylation of these tails correlates directly with the levels of active transcription from a promoter [Narlikar *et al.*, 2002]. Several transcription factors and coactivators are known to possess histone acetyltransferase (HAT) activity directly (e.g. the p300/CBP coactivators), or to recruit factors that do. Various cognate transcriptional repressors have histone deacetylase (HDAC) function. Moreover, silencing of transgene in cells transduced with AAV vector has been shown to involve HDAC activity and chromatin condensation, and to be reversible upon the addition of an HDAC inhibitor [Chen *et al.*, 2000]. Correspondingly, Yamano *et al.* [Yamano *et al.*, 2000] were able to prevent transgene silencing by co-administering a soluble HDAC inhibitor in conjunction with their plasmid vector. Remarkably, reporter transgene expression was enhanced by 50-fold in a mouse melanoma cell line and 5200-fold in NIH3T3 cells *in vitro* when drug was co-administered with plasmid vector. When delivered by intratumoural injection *in vivo*, expression could be enhanced by a factor of 3-4-fold. These studies indicate that the incorporation of such factors, whether genetically or physically, may have considerable utility in improving expression profiles from both episomal and integrating gene transfer vectors. Alternatively, the incorporation of elements able to recruit such factors may perform an equivalent function. Many constitutively active promoters are known to utilise sequences that insulate the promoter from being packaged into higher order structure and suppress PEV (LCRs) and/or facilitate attachment of the promoter to

discrete and specific transcriptionally active domains of the nuclear matrix (MARs). Indeed, these may represent one of the simplest means of ensuring a promoter remains active when introduced *in vivo*.

Locus Control Regions (LCRs)

Areas of chromatin that are transcriptionally active are denoted by an increased DNase I hypersensitivity (HS sites) and hyperacetylation. The induction and maintenance of this state is now thought to be controlled by sequence elements called LCRs, which have been identified in a large number of gene loci, including the human growth hormone [Shewchuk *et al.*, 2001], desmin [Raguz *et al.*, 1998], MCP-1 [Wagner *et al.*, 2001], and γ -globin gene locus [Forrester *et al.*, 1987]. The basic function of an LCR is to modulate chromatin structure promote transcription of either individual genes or gene clusters, though exactly how they function is still not fully understood (reviewed in [Bonifer, 2000; Festenstein *et al.*, 2000; Levings *et al.*, 2002]) (see Fig. 4).

The most in depth study of LCR function has been performed on the γ -globin gene cluster LCR on chromosome 11, which consists of 5 DNase I hypersensitive sites (HS1-5) positioned 5' to the globin genes, and is required for their transcriptional and developmental stage-specific regulation (reviewed in [Levings *et al.*, 2002]). The LCR recruits a number of transcription factors and is thought to form a 'holocomplex', which is then responsible for the modulation of chromatin structure and activation of associated genes [Johnson *et al.*, 2001]. The HS2 element of the globin LCR is also capable of modulating nuclear localization; the presence of HS2 is capable of localizing a transgene away from centromeric heterochromatin to a transcriptionally permissive nuclear region [Francastel *et al.*, 1999]. The human growth hormone LCR is similarly able to induce the formation of a 32kb hyperacetylated domain that

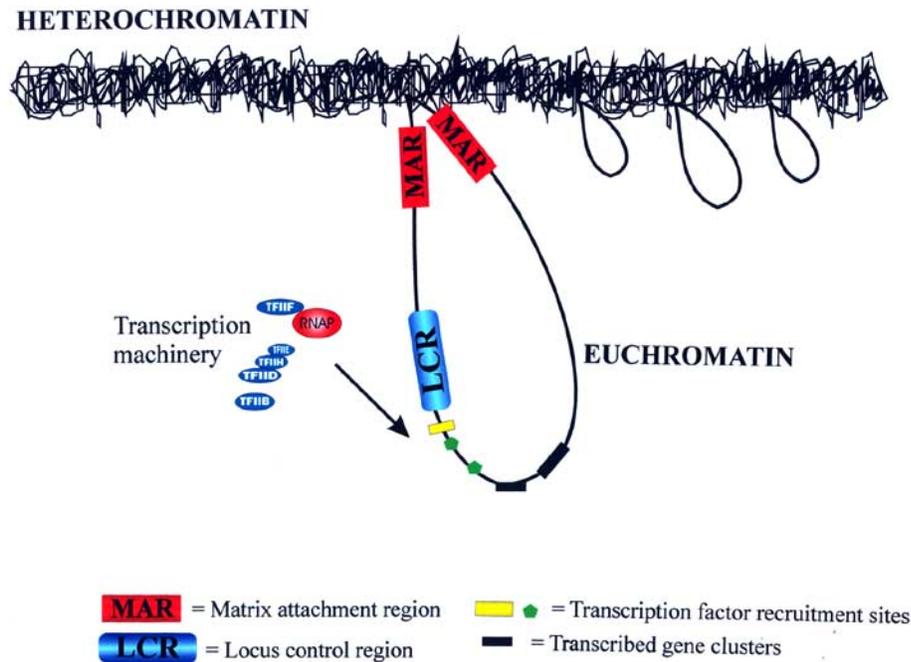


Fig. (4). A schematic illustration of the function of MAR and LCR elements in orchestrating chromosomal organisation. Chromatin remodelling factors recruited to these elements (see Fig. 3) open up areas of dense heterochromatin into areas of actively transcribed euchromatin that are accessible to the transcription machinery.

encompasses the contiguous promoter [Elefant *et al.*, 2000; Ho *et al.*, 2002].

It would be an obvious advantage to be able to harness the ability of an LCR to modulate the chromatin structure for any gene transfer vector where long-term expression is required. LCR elements have been used in a number of studies with the aim of enhancing and prolonging transgene expression. A combination of HS 2, 3 and 4 from the globin LCR was shown to endow an episomal plasmid vector with the ability to maintain stable transgene expression in the absence of drug selection for 60 days (more than 60 cell replications) [Chow *et al.*, 2002]. Miao *et al.* [Miao *et al.*, 2000; Miao *et al.*, 2001] also incorporated the apolipoprotein E LCR into episomal plasmid vectors and showed significant improvement over constructs lacking the LCR. Plasmid vectors with a combination of fragments of HS 2, 3 and 4 dramatically increased the expression in K562 (17 fold) and MEL cells (94 fold) [Emery *et al.*, 1998], although the hybrid LCR caused some instability in a retroviral vector. Kowolik *et al.* [Kowolik *et al.*, 2001] showed that HIV based lentiviral vectors were much more stable as compared to MLV-based retroviral vectors for LCR insertion. They found that the human CD2 LCR sequence is compatible with HIV vector sequences and confers enhanced integration site-independent and copy number-dependent expression of the transgene. This was also demonstrated by May *et al.* [May *et al.*, 2000], where sustained therapeutic levels of β -globin were achieved *in vivo* from a lentiviral vector containing the core elements from HS 2, 3 and 4. HIV-based lentiviral

vectors may therefore, provide a more stable platform to deliver genes controlled by various *cis*-acting elements, such as LCRs. When used in transgenic animals, the globin LCR is able to confer an open and accessible chromatin structure at ectopic sites and is able to prevent the strong position-of-integration effects (position effect variegation) observed in the absence of the LCR [Cranston *et al.*, 2001].

Matrix Attachment Regions (MARs)

Eukaryotic chromosomes are typically arranged as 50-to 100-kb loop domains that are attached at their bases to the intranuclear framework by non-histone proteins. Matrix attachment regions (MARs; or scaffold attachment regions 'SARs') are sequence elements that have been found to co-localise with transcriptional enhancers, promoters and origins of replication, and define the borders between chromatin domains (harbouring a functional segment of a gene, gene, or gene cluster) [Durrin *et al.*, 2002] (see Fig. 4). Structurally, they usually comprise AT-rich sequences of high unwinding propensity [Bode *et al.*, 1992] and enforce a curved DNA structure [Yamamura *et al.*, 2001] (a program for the identification of potential MARs is available at <http://www.futuresoft.org/modules/MarFinder/index.html>). Several MAR-DNA binding proteins have been isolated which mediate the attachment of chromatin to nuclear protein structures (some of which are cell-type specific) and the transcription machinery [Chattopadhyay *et al.*, 2000; Chattopadhyay *et al.*, 1998; Dickinson *et al.*, 1997; Durrin *et al.*, 2002; Nayler *et al.*, 1998; Neugebauer *et al.*, 1997].

Moreover, Piechaczek *et al.* [Piechaczek *et al.*, 1999] demonstrated that inclusion of a MAR in a plasmid vector led to its maintenance *in vitro* as a stable episome over more than 100 generations without any selective pressure, a finding that may also have significance for episomal Ad vectors.

Schiedner *et al.* [Schiedner *et al.*, 2002] identified an 800bp region by computer analysis conforming to all the criteria for a strong MAR within the first intron of the human α -1 anti-trypsin gene. A 2kb fragment containing this region increased gene expression 10-fold when introduced in an HCAAd vector genome carrying a phosphoglycerate kinase (PGK) promoter/ hAAT cDNA construct. The inclusion of a 415bp MAR from immunoglobulin- λ (Ig λ) in a lentiviral vector also significantly increased the human AAT and human FIX production *in vitro* (two to four-fold) and also increased the transduction efficiency of the vector [Park *et al.*, 2001]. Another study was unable to demonstrate any benefit to be gained by the inclusion of a MAR, but this may have been because it was redundant in a vector that already incorporated a strong eukaryotic promoter, intron and LCR [Ehrhardt *et al.*, 2002].

Chromatin Insulators

Chromatin insulators have been found in a large number of vertebrate and invertebrate gene loci between genes with independent profiles of expression (reviewed in [Sun *et al.*, 1999; Udvardy, 1999; Zhan *et al.*, 2001]). They function to prevent inappropriate interactions between the regulatory elements of neighbouring gene loci. Chromatin insulators have been defined by their ability to block the effects of enhancers, and by preventing insertional position effects seen by randomly integrated transgenes. It has been observed that some MARs may have insulating activity (e.g. the lysozyme MAR [Wells *et al.*, 1999]) in an enhancer-blocking assay, although there are a number of other insulators that do not have MAR activity [Zhan *et al.*, 2001]. It is also possible that insulators may interact with MAR elements through different proteins *in vivo*, with independently regulated genes or clusters of genes being contained in the individual chromatin loops anchored on the nuclear scaffold [Zhan *et al.*, 2001]. The relationship between insulators and LCRs is also poorly defined; the HS5 of β -globin LCR also contains an insulator [Li *et al.*, 2002] as does cHS4 from the chicken β -like globin gene cluster [Rivella *et al.*, 2000], which also contains significant LCR function. Even an actively transcribed promoter has been shown to have 5' insulating properties (Drosophila *eve* promoter [Zhan *et al.*, 2001]). To date these elements have been primarily studied as distinct entities, however their interactions *in vivo* may prove to be more intrinsically linked.

Isolated insulator elements have been successfully used in a number of viral vector systems to either insulate the expression cassette from other elements in the vector backbone, or to insulate integrating vectors from position effects. The 1.2kb cHS4 was inserted into the MoMLV long terminal repeat (LTR) without any adverse effects on vector production, and insulated integrated proviral DNA from insertional position effects (and also dramatically decreased

the level of *de novo* methylation of the 5' LTR) [Rivella *et al.*, 2000]. Steinwaerder *et al.* [Steinwaerder *et al.*, 2000] inserted an expression cassette driven by a metal (Zn²⁺) inducible promoter into an adenoviral vector, and observed that the background transgene expression increased significantly as a result of *cis*-acting viral sequences. Flanking the expression cassette by the cHS4 sequence increased the induction ratio by 15-fold *in vivo*. In an AAV vector, a 42 bp tandem repeat of sequence from cHS4 also gave a modest reduction in background transcription using a tetracycline inducible promoter caused by the ITRs. Size restraints prevented any larger fragments being used [Fitzsimons *et al.*, 2001]. Other regulable switches may also benefit from insulation in viral vectors to reduce transactivating interference from flanking viral sequences.

RNA Processing and Structural Moieties

It has become apparent that the processes of post-transcriptional processing are tightly coupled to transcription. Transcript capping, splicing and polyadenylation are now understood to occur co-transcriptionally, and many of the factors executing these processes interact with the elongating RNAP_{II} and influence the process of transcription by feedback control (reviewed in [Hirose *et al.*, 2000]). All of these mechanisms represent potential rate limiting steps in transgene expression and have correspondingly significant implications for the design of expression cassettes in gene therapy. It is therefore, essential that gene expression cassettes not only contain optimal elements for modulating chromatin structure and a promoter that will provide persistent gene expression, but also consider that sub-optimal splicing, polyadenylation or the deletion of untranslated regions (UTRs) may affect transgene production adversely.

Several studies help to illustrate this point. Miao *et al.* [Miao *et al.*, 2000] produced a series of plasmid constructs combining the apolipoprotein E locus control region, hAAT promoter, human factor IX minigene (hFIXmg) sequence including a portion of the first intron (intron A), 3'untranslated region (3'UTR), and a bovine growth hormone polyadenylation signal (bGH P(A)). They found that this plasmid gave a 2-fold increase in factor IX expression *in vitro*, however, following systemic administration *in vivo* the difference in expression was up to 65-fold. Plasmids containing no intron sequences resulted in low to undetectable levels and transient gene expression *in vivo*. However, plasmids containing the combination of ApoE-LCR and hAAT promoter, an intron, and polyadenylation signal(s) produced persistent, therapeutic levels (0.5–2 μ g/ml) of hFIX. Plasmids containing a 3' UTR gave ~2-fold higher expression than those containing the bGH P(A) alone. Furthermore, vectors without the ApoE-LCR sequences produced a lower level of initial gene expression, and the level became undetectable 4-5 weeks after plasmid injection, indicating that ApoE-LCR was an essential element for persistent hFIX gene expression. Erhardt and Kay [Ehrhardt *et al.*, 2002] similarly demonstrated the role played by these elements *in vivo*. Systemically administered first-generation Ads incorporating genomic control elements, including an intron and LCR, and driven by the hAAT promoter were able to induce sustained

levels of factor IX at up to 100µg/ml. Notably, expression from the vector retaining a 3'UTR persisted at high level for over 9 weeks, whilst that from vectors lacking this element fell by 40-fold.

Xu *et al.* [Xu *et al.*, 2001] tested the SV40 P(A) signal and compared it to the bGH P(A) and minimal rabbit -globin P(A) in plasmid vectors. The different P(A) sequences lead to 2-3 fold differences in gene expression in a number of cell lines *in vitro* and tissues *in vivo*. However, the most efficient P(A) sequence varied in the different situations tested. The effect of adding intron A (the largest intron of the human CMV IE gene) showed modest benefits, increasing expression by 2 to 6-fold *in vitro* and 1.5 to 3-fold *in vivo*. Following this, the same constructs were used to generate Ad vectors [Xu *et al.*, 2002] with a much greater differential in the results. Intron A insertion generated a 2- to 20-fold higher level of luciferase expression *in vitro* and a 10-fold increase in expression *in vivo* in multiple tissues types, with a 50-fold increase seen in the liver. bGH P(A), rather than being weaker *in vitro* and in mouse muscle when compared to SV40 P(A), displayed higher activity than SV40 P(A) both *in vitro* and *in vivo*, by at least a factor of two. These studies reveal the need to empirically test a number of elements *in vitro* and *in vivo*.

Another RNA element that has shown to enhance protein expression is the woodchuck hepatitis virus (WHV) post-translationally regulated element (WPRE) [Donello *et al.*, 1998], which spans approximately 600bp of the WHV genome. Whilst its mode of action is still uncertain, studies with WPRE and other related sequences suggests an enhancement of RNA polyadenylation, RNA export, and RNA translation [Donello *et al.*, 1998; Loeb *et al.*, 1999; Schambach *et al.*, 2000]. The increase in protein expression in a wide range of different cell types and vectors has been shown to promote a 2-fold upregulation of protein expression: AAV vectors [Fitzsimons *et al.*, 2001], lentiviral vectors (1.5-3 fold) [Ramezani *et al.*, 2000], SIN lentiviral vectors [Moreau-Gaudry *et al.*, 2001] and Ad vectors [Glover *et al.*, 2002]. Two studies have shown an increase of approximately 7 fold in protein expression: Loeb *et al.* [Loeb *et al.*, 1999] with AAV vectors on 293 cells and primary fibroblasts showed a 6-8 fold increase (3-4 fold increase in mRNA), and Schambach *et al.* [Schambach *et al.*, 2000] saw a 7 fold increase with retroviral vectors. Some final other RNA processing considerations include avoiding the formation of double stranded RNA (that may cause RNA interference [Riddihough, 2002]), avoiding AUUUA sequences in the 3'UTR (this sequence is found in genes that are rapidly turned over), and avoiding the inclusion of introns after the stop codon, which may dramatically reduce expression.

Summary

Our increased understanding of gene regulation *in vivo* has revealed important considerations for the expression of genes for biological studies and gene therapy. Information now available on the mechanisms and regulation of transcription, translation and the coordinated organisation of chromatin structure has yet to be incorporated into the design of many commonly used vector constructs. The application

of this knowledge in the development of specific 'tailor-made' expression cassettes should enable the realisation of the high-level and persistent transgene expression required for many gene therapy applications.

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