HHS Public Access

Author manuscript

Discov Med. Author manuscript; available in PMC 2015 July 17.

Published in final edited form as:

Discov Med. 2015 January ; 19(102): 49–57.

Viral Expression Cassette Elements to Enhance Transgene Target Specificity and Expression in Gene Therapy

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Abstract

Over the last five years, the number of clinical trials involving AAV (adeno-associated virus) and lentiviral vectors continue to increase by about 150 trials each year. For continued success, AAV and lentiviral expression cassettes need to be designed to meet each disease's specific needs. This review discusses how viral vector expression cassettes can be engineered with elements to enhance target specificity and increase transgene expression. The key differences relating to target specificity between ubiquitous and tissue-specific promoters are discussed, as well as how endogenous miRNAs and their target sequences have been used to restrict transgene expression. Specifically, relevant studies indicating how *cis*-acting elements such as introns, WPRE, polyadenylation signals, and the CMV enhancer are highlighted to show their utility for enhancing transgene expression in gene therapy applications. All discussion bears in mind that expression cassettes have space constraints. In conclusion, this review can serve as a menu of vector genome design elements and their cost in terms of space to thoughtfully engineer viral vectors for gene therapy.

Introduction

The *cis*-acting elements that regulate transgene expression can have as great of an impact on the success of gene therapy as the design of the vector capsid or envelope. Target specificity and an appropriate level of transgene expression can prevent unwanted phenotypes in other cells, an immune response, and possible toxicity. Overexpression and non-targeted expression in some diseases, such as Rett Syndrome, is to be avoided (Amir *et al*., 1999); however, in Hemophilia B, expression of Factor IX, a secreted protein present in the blood, is needed to be high and there is little concern of overexpression (reviewed in Cancio *et al*., 2013).

Lentivirus and AAV (adeno-associated virus) expression cassettes, prominently used in gene therapy, can be designed for target specificity and transgene expression levels (Figure 1). Target specificity can be honed by using cell-specific promoters or endogenous miRNAs.

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Disclosure The authors have no conflicts of interest to disclose.

Transgene expression levels can be modulated by engineering the expression cassette to include the CMV enhancer (that includes transcription factor binding sites) or mRNA stability/nuclear export *cis*-acting elements (introns, polyA signals, or WPRE). Expression cassettes require thoughtful design due to foreign DNA packaging size constraints of AAV and lentivirus, approximately 4.1–4.9 kbs and 8–9 kbs, respectively (Dong *et al*., 1996; Kumar *et al*., 2001). While keeping in mind size constraints, this review will discuss different *cis*-acting elements that have been engineered into lentivirus and AAV expression cassettes to enhance cell-specific transgene expression. Lentivirus and AAV have been extensively reviewed elsewhere in the areas of their pros and cons, virology, uses, and development for gene transfer (Nagabhushan Kalburgi *et al*., 2013; Kay *et al*., 2011; Grieger and Samulski, 2012; Segura *et al*., 2013). Other outstanding reviews are available for insulators (Antoniou *et al*., 2013), self-complementary AAV (McCarty, 2008), AAV serotype tropism (Wu *et al*., 2006), retrovirus pseudotyping (Matrai *et al*., 2010), and systems to induce/regulate expression using exogenously supplied *trans*-acting factors (Toniatti *et al*., 2004). Although these are useful tools to control expression and/or cell specificity, they will not be discussed in this review. Moreover, while the genome modifications are described in this review in the context of AAV and lentiviral vectors, they are certainly applicable to other vector systems.

Promoters

An effective gene transfer approach must be directed to the specific tissues/cells where it is needed, and the resulting transgene expression should be at a level that is appropriate to the specific application. Promoters are a major *cis*-acting element within the vector genome design that can dictate the overall strength of expression as well as cell-specificity (Table 1).

Ubiquitous expression

In some cases, such as those where a gene product is secreted, ubiquitous expression in all cell types is desired. Constitutive promoters such as the human elongation factor 1α-subunit (EF1α), immediate-early cytomegalovirus (CMV), chicken β-actin (CBA) and its derivative CAG, the β glucuronidase (GUSB), or ubiquitin C (UBC) can be used to promote expression in most tissues (Husain *et al*., 2009; Qin *et al*., 2010; Norrman *et al*., 2010). Generally, CBA and CAG promote the larger expression among the constitutive promoters (Xu *et al*., 2001; Yin *et al*., 2011); however, their size of ~1.7 kbs in comparison to CMV (~0.8 kbs) or EF1α (~1.2 kbs) limits its use in vectors with packaging constraints such as AAV. The GUSB or UBC promoters can provide ubiquitous gene expression with a smaller size of 378 bps and 403 bps, respectively, but they are considerably weaker than the CMV or CBA promoter (Husain *et al*., 2009; Qin *et al*., 2010). Thus, modifications to constitutive promoters in order to reduce the size without affecting its expression have been pursued and examples such as the CBh (~800 bps) and the miniCBA (~800 bps) can promote expression comparable and even higher in selected tissues (Gray *et al*., 2011). It should be noted that in some cases “ubiquitous” promoters can be prone to silencing or promote differential expression strength in selected cell types (McCown *et al*., 1996; Klein *et al*., 1998; Gray *et al*., 2011).

Tissue-specific expression

When expression should be restricted to certain cell types within an organ, promoters can be used to mediate this specificity. For example, within the nervous system promoters have been used to restrict expression to neurons, astrocytes, or oligodendrocytes. In neurons, the neuron-specific enolase (NSE) promoter drives stronger expression than ubiquitous promoters (Xu *et al*., 2001); however, its size of 2.2 kbs limits its use in smaller vectors. Additionally, the platelet-derived growth factor B-chain (PDGF-β), the synapsin (Syn), and the methyl-CpG binding protein 2 (MeCP2) promoters can drive neuron-specific expression at lower levels than NSE, but their sizes of 1.4 kbs, 470 bps and 229 bps, respectively, make them more suitable for vectors with limitations in size (Paterna *et al*., 2000; Kügler *et al*., 2003; Hioki *et al*., 2007; Kuroda *et al*., 2008; Rastegar *et al*., 2009; Gray *et al*., 2011). In astrocytes, the 680 bps-long shortened version [gfaABC(1)D] of the glial fibrillary acidic protein (GFAP, 2.2 kbs) promoter can confer higher levels of expression with the same astrocyte-specificity as the GFAP promoter (Lee *et al*., 2008). Targeting oligodendrocytes can also be accomplished by the selection of the myelin basic protein (MBP) promoter, whose expression is restricted to this glial cell; however, its size of 1.9 kbs and low expression levels limit its use (Chen *et al*., 1998).

Following systemic administration of vectors, cell- or tissue-specific promoters can be used to restrict expression away from the liver. In skeletal muscle cells, the promoters based on muscle creatine kinase (MCK) and desmin (1.7 kbs) have showed a high rate of specificity with minimal invasion to the liver (Wang *et al*., 2008; Talbot *et al*., 2010; Katwal *et al*., 2013). The promoter of the α-myosin heavy chain (α-MHC; 1.2 kbs) has shown significant cardiac specificity in comparison with other muscle promoters (Lee *et al*., 2011). In hematopoietic stem cells the synthetic MND promoter (Li *et al*., 2010) and the promoter contained in the 2AUCOE (ubiquitous chromatin opening element) have shown to drive a higher transgene expression in all cell lineages when compared to the EF1α and CMV promoters, respectively (Zhang *et al*., 2007; Koldej 2013; Dighe *et al*., 2014). Conversely, using promoters to restrict expression to only liver hepatocytes after vector-mediated gene transfer has been shown to avoid transgene-specific immune responses, and to even induce immune tolerance to the expressed protein (Zhang *et al*., 2012). The α1-antitrypsin (hAAT; 347 bps) and the thyroxine binding globulin (TBG; ~400 bps) promoters drive gene expression restricted to the liver with minimal invasion to other tissues (Yan *et al*., 2012; Cunningham *et al*., 2008).

Tissue specific promoters provide the advantage of limiting the expression to the desired cell or tissue. However, low levels of expression and/or large size may limit their use. To compensate for weak strength, the level of expression can be increased by adding enhancer elements such as from CMV (see below). Conversely, as mentioned above, these promoters can be modified in order to reduce their capabilities and overall strength.

Endogenous MicroRNAs

MicroRNAs (miRNAs) are 21–23 oligonucleotide RNA molecules that control protein expression by repressing genes post-transcriptionally in a tissue-, cell-, developmental-, or metabolic-specific manner (reviewed in Broderick and Zamore, 2011). Endogenous

miRNAs can `de-target' or inhibit transgene expression when their exact complementary target sequences are engineered into an expression cassette. The level of repression, *in vitro*, correlates with the number of target sequences within the expression cassette (Doench *et al*., 2003; Brown *et al*., 2006; 2007). As an example, 4 copies of the hematopoietic-specific miR-142-3p target sequence (miR-142-3pT) were engineered into a lentivirus vector with a reporter transgene being driven by the ubiquitous PGK (phosphoglycerate kinase) promoter (Brown *et al*., 2006). In fact, miR-142-3p was still able to maintain expression inhibition even if cells were overloaded with up to 30 viral genomes per cell (Brown *et al*., 2006). The miR-142-3pT containing viruses, when injected intravenously into mice, inhibited transgene expression in Kupffer cells and restricted transgene expression to hepatocytes and liver endothelial cells (Brown *et al*., 2006). Transgene expression was further restricted to only liver endothelial cells, when 4 copies of miR-142-3pT and 4 copies of miR-122aT were combined within an expression cassette (Brown *et al*., 2007). In another *in vivo* study, when an engineered lentiviral vector containing 4 copies of the neuronal-specific miR-124 target sequence was injected into mouse brain, PGK-driven transgene expression was de-targeted from neurons to only astrocytes (Colin *et al*., 2009). Endogenous miRNAs are a useful tool in obtaining transgene cell specificity because their respective binding sites are small, can be combined, and are robust in their ability to restrict expression.

Post-transcriptional Regulatory Elements

Viral post-transcriptional regulatory elements (PREs) are important for viral gene expression; these *cis*-acting elements are required for nuclear export of intronless viral RNA (Huang and Yen, 1994; 1995). Both HPRE (Hepatitis B Virus PRE, 533 bps) and WPRE (Woodchuck Hepatitis Virus PRE, 600 bps) were assessed, *in vitro*, and the level of transgene expression was increased 6.1-fold and 8.6-fold, respectively (Donello *et al.*, 1998). The difference in expression was determined to be due to an additional sequence element in WPRE (Donello *et al.*, 1998). WPRE can be shortened (to 247 bps), as demonstrated in neurons *in vivo* and *in vitro*, and it still offers sufficient transgene expression (Choi *et al.*, 2014). In cultured human cells using lentiviral and AAV vectors, WPRE was found to increase CMV promoter driven transgene expression up to 8-fold (Loeb *et al.*, 1999; Zufferey *et al.*, 1999). *In vivo* studies have also shown an increase of PPE, PDGF, NSE, or CMV promoter-driven transgene expression by the presence of WPRE (Paterna *et al.*, 2000; Xu *et al.*, 2001). Importantly, transgene expression was not significantly increased by including WPRE, *in vitro* and *in vivo*, when driven from either the EFα1 or CAG promoter due to an intron in the promoters (Ramezani *et al.*, 2000; Fagoe *et al.*, 2014). Another effect of the WPRE is to protect transgenes from silencing, as seen when it was combined with the CMV or CAG promoter in human ES cells and in the brain (Paterna *et al.*, 2000; Xia *et al.*, 2007). In conclusion, although the WPRE can boost expression and prevent long-term silencing in combination with several promoters, the presence of an intron seems to mitigate its effectiveness in boosting transgene expression levels.

Polyadenylation Signal Sequences and Upstream Enhancer

The polyadenylation of a transcript is critical for nuclear export, translation, and mRNA stability. Therefore, the efficiency of transcript polyadenylation is important for transgene expression. *In vitro* studies using mammalian cultured cells have been useful in determining the effects of different polyA signals to boost expression. One study, in human epithelial-like cells, found that a transgene had a 2.5-fold increase in expression with either SV40 late or bovine growth hormone polyA (bGHpA) signal sequences compared to a minimal synthetic polyA (SPA) signal (Levitt *et al.*, 1989; Yew *et al.*, 1997). Some of the same polyA signals were assessed in neuronal cell cultures and gave similar results; the late SV40 polyA signal and bGHpA were approximately equivalent and twice as strong as the minimal SPA (Choi *et al.*, 2014). *In vivo*, the bGHpA signal, when packaged into AAV2 and injected intravenously into mice, gave 2- to 3-fold more transgene expression over the mouse β-globin polyA signal (Wu *et al.*, 2008). Together these results suggest that polyA signal strength is independent of cell type and that *in vitro* results generally correlate with *in vivo* observations.

The efficiency of polyadenylation is increased by the SV40 late polyA signal upstream enhancer (USE) placed upstream of other polyA signals (Schek *et al.*, 1992). The SV40 late + 2xUSE polyA signal compared to SV40 late polyA signal alone gave about a 2-fold increase in transgene expression (Schambach *et al.*, 2007; Choi *et al.*, 2014). SV40 late +2xUSE polyA signal also increased transgene expression by 45–100% when compared to a variety of other USEs (Schambach *et al.*, 2007). *In vivo*, bGHpA and SV40 late +2xUSE polyA signals, when injected into mouse hippocampus, gave similar levels of increased transgene expression compared to the control (Choi *et al.*, 2014). Interestingly, a study comparing SV40 late +2xUSE polyA signal and a shortened WPRE (247 bps) to bGHpA and WPRE found that both increased transgene expression to a similar level; however, the first construct is about 400 bps shorter (Schambach *et al.*, 2007). These results are summarized in Table 2.

CMV Enhancer

The CMV enhancer is upstream of the CMV promoter at −598 to −68 (Boshart *et al.*, 1985) (~600 bps) and contains transcription binding sites. In cultured cells, the presence of the CMV enhancer increased tissue-specific promoter-driven transgene expression 4-, 8-, 45-, and 90-fold in cardiomyocytes using the ANF (atrial natriuretic factor) promoter, in mouse and human epithelial cells using the CC10 (club cell 10) promoter, in lung epithelial cells using the SP-C (surfactant protein C) promoter, and in neurons using the PDGF-β (platelet-derived growth factor-β) promoter, respectively (Yew *et al.*, 1997; Liu, B. *et al.*, 2004; Gruh *et al.*, 2008). Strikingly, in neuronal cell culture, the CMV enhancer and tissue-specific promoter drove transgene expression levels as strong as the CMV enhancer and promoter (Liu *et al.*, 2004). *In vivo* mouse studies, using a modified AAV2 intravenously injected into mice, found that using the CMV enhancer upstream of a cardiac muscle promoter resulted in 50-fold more transgene expression in the heart than with the CMV promoter alone (Muller *et al.*, 2006). Also in AAV, *in vivo* when injected directly into muscle, transgene expression using the CMV enhancer with a synthetic muscle-specific promoter (C5-12) was similar to

the CMV promoter level and 50% more than the C5-12 promoter alone (Liu *et al.*, 2004). Together, the CMV enhancer increases transgene expression under different cell-specific promoters and different cell types making it a broadly applicable tool to increase transgene expression levels.

Introns

The presence of an intron or intervening sequence in mRNA was first described, *in vitro*, to be important for mRNA processing and increased transgene expression (Huang and Gorman, 1990; Niwa *et al.*, 1990). Early *in vitro* comparison studies indicated that the SV40 intron did not increase transgene expression in mouse lung epithelial cells when placed between the promoter and transgene, while a hybrid intron (adenovirus/mouse immunoglobulin) increased transgene expression by 1.6-fold (Yew *et al.*, 1997). However, the presence of the SV40 intron between the promoter and the transgene, in an AAV expression cassette, gave a 2-fold increase of transgene expression under the CMV promoter and enhancer in lung carcinoma cells (Ostedgaard *et al.*, 2005). A variety of introns (Table 3) placed between the promoter and transgene were compared, in mice using AAV2, for liver transgene expression (Wu *et al.*, 2008). The MVM (minute virus of mice) intron increased transgene expression more than any other intron tested and more than 80-fold over no intron (Wu *et al.*, 2008). However, in cultured neurons using AAV expression cassettes, transgene expression was less under a CaMPKII promoter with a chimeric intron (human β-globin donor and immunoglobulin heavy chain acceptor) between the transgene and polyA signal compared to a WPRE (Choi *et al.*, 2014). Together, an intron can be a valuable element to include in an expression cassette to increase transgene expression.

Summary

AAV and lentiviral expression cassettes for gene therapy can be engineered to enhance transgene target specificity and expression. The specificity of transgene expression can be controlled using cell-specific promoters and endogenous miRNAs. The overall strength of expression can be increased up to 90-fold with the CMV enhancer or up to 80-fold by improving mRNA stability/nuclear export with a WPRE, polyA signal, an USE, or an intron. The combination of these elements must be given thoughtful consideration in order to adhere to the space constraints of AAV and lentivirus vectors for gene therapy.

Acknowledgments

Indirect support was provided by Research to Prevent Blindness to the UNC Department of Ophthalmology. Support for R. Rivera-Soto was provided by the UNC Post-Baccalaureate Research Education Program (PREP) and S.K. Powell was supported in part by NS082289 to Thomas McCown.

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Figure 1.

Cartoon diagram of a generic AAV or lentiviral expression cassette design indicating where modular regulatory elements would be placed. The promoter, ITR (inverted terminal repeats)/LTR (long terminal repeats), and polyA are essential. The other elements are optional. CE, CMV enhancer; I, intron; W, WPRE; M, miRNA target sequences; U, polyA upstream enhancer; pA, polyA signal.

Table 1

Comparison of Selected Ubiquitous and Cell-specific Promoters.

Promoter	Specificity	Relative Strength	Size (bps)	Reference(s)
CMV	Ubiquitous	+++	750–800	Xu *et al.*, 2001; Gray *et al.*, 2011
CBA (including derivatives: CAG, CBh, etc.)	Ubiquitous	+++	248–1,600	Klein *et al.*, 2002; Ohlfest *et al.*, 2005; Gray *et al.*, 2011
EF-1α	Ubiquitous	++	2,500	Gill *et al.*, 2001; Xu *et al.*, 2001; Ikeda *et al.*, 2002; Gilham *et al.*, 2010
PGK	Ubiquitous	++	426	Gilham *et al.*, 2010
UBC	Ubiquitous	+	403	Gill *et al.*, 2001; Qin *et al.*, 2010
GUSB (hGBp)	Ubiquitous	+	378	Husain *et al.*, 2009
UCOE (Promoter of HNRPA2B1-CBX3)	Ubiquitous	++	600–2,500	Antoniou *et al.*, 2013
hAAT	Liver	++	347–1,500	Van Linthout *et al.*, 2002; Cunningham *et al.*, 2008
TBG	Liver	++	400	Yan *et al.*, 2012
Desmin	Skeletal muscle	+++	1,700	Talbot *et al.*, 2010
MCK	Skeletal muscle	++	595–1,089	Wang *et al.*, 2008; Talbot *et al.*, 2010; Katwal *et al.*, 2013
C5-12	Skeletal, cardiac, and diaphragm	++	312	Wang *et al.*, 2008
NSE	Neuron	+++	300–2,200	Xu *et al.*, 2001
Synapsin	Neuron	+	470	Kügler *et al.*, 2003; Hioki *et al.*, 2007; Kuroda *et al.*, 2008
PDGF	Neuron	+++	1,400	Patterna *et al.*, 2000; Hioki *et al.*, 2007
MecP2	Neuron	+	229	Rastegar *et al.*, 2009; Gray *et al.*, 2011
CaMKII	Neuron	++	364–2,300	Hioki *et al.*, 2007; Kuroda *et al.*, 2008
mGluR2	Neuron	+	1,400	Brené *et al.*, 2000; Kuroda *et al.*, 2008
NFL	Neuron	+	650	Xu *et al.*, 2001
NFH	Neuron	+	920	Xu *et al.*, 2001
nβ2	Neuron	+	650	Xu *et al.*, 2001
PPE	Neuron	+	2,700	Xu *et al.*, 2001
Enk	Neuron	+	412	Xu *et al.*, 2001
EAAT2	Neuron and astrocyte	++	966	Su *et al.*, 2003; Kuroda *et al.*, 2008
GFAP	Astrocyte	++	681–2,200	Brenner *et al.*, 1994; Xu *et al.*, 2001; Lee *et al.*, 2008; Dirren *et al.*, 2014
MBP	Oligodendrocytes	++	1,900	Chen *et al.*, 1998

Note: Cell type specificity, relative strength (+ being the weakest and +++ being the strongest), size, and relevant references for commonly used promoters.

Table 2

Comparison of PolyA Signals and USEs.

PolyA Signal and USE	Relative Strength	Size (bps)	Source	Reference(s)
hGH	+	624	Human growth hormone	Ostedgaard *et al*., 2005
SV40 late	+++	135	Simian virus 40	Choi *et al*., 2014
SPA (synthetic polyA)	+	49	Rabbit β-globin	Levitt *et al*., 1989; Yew *et al*., 1997; Ostedgaard *et al*., 2005; Choi *et al*., 2014
bGH	++	250	Bovine growth hormone	Yew *et al*., 1997; Xu *et al*., 2001; Wu *et al*., 2008; Gray *et al*., 2011; Choi *et al*., 2014
SV40 late 2xUSE	++	100	Simian virus 40	Schambach *et al*., 2007; Choi *et al*., 2014
HIV-1 USE	+	35	Human immunodeficiency virus 1	Schambach *et al*., 2007
GHV USE	+	39	Ground squirrel hepatitis virus	Schambach *et al*., 2007
Adenovirus (L3) USE	+	21	Adenovirus	Schambach *et al*., 2007
hTHGB USE	+	21	Human prothrombin	Schambach *et al*., 2007
hC2 USE	+	53	Human C2 complement gene	Schambach *et al*., 2007

Note: The relative strength (+ being the weakest and +++ being the strongest), source, size, and relevant references for each polyA signal or USE is listed.

Table 3

Comparison of Introns.

Intron	Relative Strength	Size (bps)	Source	Reference(s)
MVM	+++	67–97	Minute virus of mice	Wu *et al.*, 2008
F.IX truncated intron 1	+	300	Human factor IX	Wu *et al.*, 2008; Kurachi *et al.*, 1995
β-globin SD / immunoglobin heavy chain SA	+	250	Human, pZac2.1	Wu *et al.*, 2008; Choi *et al.*, 2014
Adenovirus SD# / immunoglobulin SA*	++	500	pAdβ	Wong *et al.*, 1985; Yew *et al.*, 1997
SV40 late SD# / SA* (19S/16S)	+	180	pCMVβ	Yew *et al.*, 1997
Hybrid adenovirus SD# / IgG SA*	+++	230	Adenovirus	Choi *et al.*, 1991; Huang and Gorman, 1990

Note: The relative strength (+ being the weakest and +++ being the strongest), source, size, and relevant references for each intron is listed. SD#, splice donor; SA*, splice acceptor.