Chapter 67
Distinct Expression Patterns of AAV8 Vectors with Broadly Active Promoters from Subretinal Injections of Neonatal Mouse Eyes at Two Different Ages

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Abstract The retinal expression patterns were analyzed following the injection of serotype 8 adeno-associated virus (AAV8) vectors that utilize two broadly active and commonly used sets of transcription regulatory sequences. These include the human cytomegalovirus (CMV) immediate early (IE) enhancer/promoter and the hybrid CAG element (also known as CAGGS or CBA) composed of a partial human CMV IE enhancer and the chicken β-actin promoter and intron. Subretinal delivery to postnatal day 0 (P0) or 6 (P6) mouse eyes resulted in efficient labeling of retinal cells, but with very distinct patterns. With P0 delivery, AAV8-CMV-GFP selectively labelled photoreceptors, while AAV8-CAG-GFP efficiently labeled both outer and inner retinal neurons, including photoreceptors, horizontal cells, amacrine cells and retinal ganglion cells. With P6 delivery, both vectors led to efficient labeling of photoreceptors and Müller glia cells, but not of inner retinal neurons. Our results suggest that the cell types that express the genes encoded by subretinally delivered AAV8 vectors are determined by both the timing of the injection and the regulatory sequences.

Keywords AAV8 · Subretinal injection · Neonatal mouse eye · Cellular tropism · Transgene expression · Human cytomegalovirus (CMV) immediate early (IE) enhancer/promoter · Chicken β-actin promoter/enhancer/intron

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67.1 Introduction

In recent years, adeno-associated virus (AAV) vectors have been widely used for ocular gene transfer. It has been shown that the labeling pattern of retinal cell types is determined by several factors, including the AAV serotype, administration route (intravitreal vs. subretinal) and timing (neonatal vs. adult), as well as the regulatory sequences used. Although cell type-specific regulatory elements of human origin are ideal for clinical applications, composite elements, such as CMV (human CMV IE enhancer/promoter/human β-globin intron) and CAG (human CMV IE enhancer/chicken β-actin promoter/intron with rabbit β-globin 3' splice site, also called the CBA or CAGGS promoter) (Boshart et al. 1985; Niwa et al. 1991), are useful for robust and long-term transgene expression in a broad range of cell types in preclinical animal studies. Here we report on the labeling patterns from these two broadly active sets of elements in AAV8 vectors following subretinal delivery at two different ages. The expression patterns from these vectors changed dramatically when they were delivered at P0 vs. P6, demonstrating that the timing of injection during neonatal eye development is an important determinant of the expression patterns within retinal cell types. These changes in expression patterns may reflect the fate of AAV genomes when delivered to mitotic vs. postmitotic cells.

67.2 Materials and Methods

67.2.1 AAV Vector Construction and Production

AAV-CMV-GFP was constructed by cloning GFP cDNA from a pCAG-GFP vector (Addgene plasmid 11150 (Matsuda and Cepko 2004)) via EcoR1/Not1 sites into an AAV-MCS8 vector, which was obtained from HMS DF/HCC DNA resource core. The AAV-CMV-GFP construct contains a human CMV enhancer/promoter, human β-globin intron, GFP cDNA, SV40 polyA signal. AAV-CAG-GFP was constructed by replacing the CMV promoter with the CAG promoter from pCAG-GFP via Spe1/EcoR1. The AAV-CAG-GFP construct contains partial human CMV IE enhancer, chicken β-actin promoter, a hybrid intron composed of a chicken β-actin 5' splice site and rabbit β-globin 3' splice site with the majority of the intron deriving from the chicken β-actin intron 1, GFP cDNA, woodchuck hepatitis virus posttranscriptional regulatory element, and SV40 polyA. AAV8 vectors were produced by triple transfection of 293T cells (AAV vector, Rep2/Cap8, and pHGTI-adeno1 helper plasmids), purified based on published method (Vandenberghe et al. 2010), titered by RT-PCR, and diluted to 5×10^{12} genome copies (gc)/ml in PBS.

67.2.2 Animals and AAV Injection

Timed pregnant E18 *CD1* animals were ordered from Charles River Laboratory. Subretinal injection of P0 and P6 eyes was performed as described (Matsuda and Cepko 2004; Wang et al. 2014). A preset volume of virus (~0.3 µl) was delivered by the Femtojet (Eppendorf).

67.2.3 Histology and Imaging

At 15 days post injection, retinas were processed for immunohistochemistry as described (Matsuda and Cepko 2004; Wang et al. 2014). Antibodies used in this study included goat anti-ChAT (Millipore, 1:100) and Cy3 anti-goat (Jackson Immuno, 1:1000).

67.3 Results

67.3.1 Distinct Patterns of GFP Expression from AAV8-CMV-GFP and AAV8-CAG-GFP Following Subretinal Injection into P0 Mouse Eyes

CMV and CAG are two commonly used broadly active sets of regulatory elements that drive robust gene expression in a broad spectrum of cell types. Although these elements have been evaluated in the past for their expression in the retina in the context of AAV vectors (Allocca et al. 2007; Watanabe et al. 2013), a comparison of the patterns following injection at P0 has not been reported. P0 subretinal injections allow for fairly uniform spread of an inoculum throughout the entire retina, presumably as the photoreceptor outer segments have not yet developed, and thus their interactions with the retinal pigment epithelium (RPE) have not created a barrier to the spread of the inoculum. We evaluated the activities of these promoters in AAV8 vectors for retina transduction (Fig. 67.1a). AAV8-CMV-GFP or AAV8-CAG-GFP vectors at a dose of $\sim 1.5 \times 10^9$ gc/eye were injected subretinally into P0 *CD1* mouse eyes, and retinas were harvested 15 days after virus injection. Bright GFP signal was observed from nearly all retinas under a dissecting fluorescent microscope, demonstrating that the early mouse retina is quite susceptible to infection and expression from these vectors (Fig. 67.1b). About 90% of the eyes examined appeared to have nearly the entire retina expressing GFP, at least some cells throughout indicating that the inoculum can indeed spread readily throughout the subretinal space when delivered at P0.

Retinal sections were processed and imaged for direct GFP fluorescence. We found that AAV8-CMV-GFP and AAV8-CAG-GFP resulted in distinct patterns of GFP expression. AAV8-CMV-GFP mainly resulted in labelled photoreceptor cells

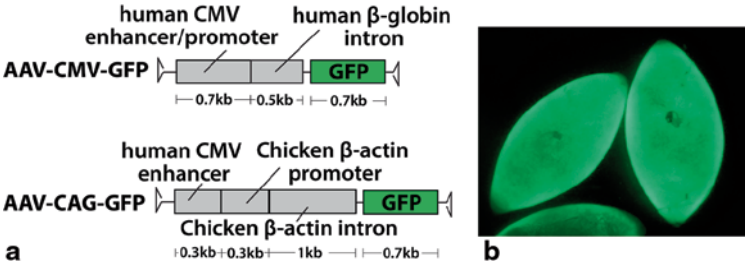

Fig. 67.1 **a** Illustrations of AAV-CMV-GFP and AAV-CAG-GFP constructs. **b** The native GFP fluorescence of the retinas transduced by AAV8-CMV-GFP with P0 subretinal injection. Retinas were harvested 15 days post infection

in the outer nuclear layer (ONL), with very few cells expressing GFP in the inner nuclear layer (INL) and ganglion cell layer (GCL) (Fig. 67.2a). In contrast, AAV8-CAG-GFP led to expression in many retinal cells in the ONL, INL, and GCL (Fig. 67.2b). In AAV8-CMV-GFP infected retinas, cones were the most efficiently and brightly labelled, while rods expressed a modest level of GFP (Fig. 67.2c). The inner, more vitreal, rods were more obviously labeled than the outer rods. In AAV8-CAG-GFP infected retinas, the cell types that expressed GFP included rods, cones, horizontal cells, amacrine cells, and ganglion cells (Fig. 67.2d). ChAT antibody staining showed that most cholinergic amacrine cells were transduced by AAV8-CAG-GFP (data not shown). As RPE cells were not included in the analysis, we cannot compare the labeling efficiency by these two vectors in the RPE. In summary, AAV8-CMV-GFP resulted in efficient labeling of photoreceptors, while AAV8-CAG-GFP provided a broader labeling pattern.

67.3.2 The Timing of Subretinal Injections in Neonatal Animals Yields Different Labelling Patterns

Next we examined the expression patterns following subretinal injection of the same AAV8 vectors at P6. At 15 days post infection, retinas were harvested and analyzed. Both vectors resulted in efficient transduction of photoreceptors and Müller glia cells, a pattern that is different from those following P0 injections (Fig. 67.2e, f).

67.4 Discussion

We found that the CMV and CAG elements drive different expression patterns of a GFP reporter in retinal cells when used in AAV8 vectors and delivered subretinally at P0. Because AAV8-CMV-GFP and AAV8-CAG-GFP vectors were packaged using the same AAV8 capsids, the different expression patterns are due to the activities of the regulatory elements. The fact that AAV8-CAG-GFP results in efficient expression of cells in the ONL, INL, and GCL demonstrates that AAV8 virions can

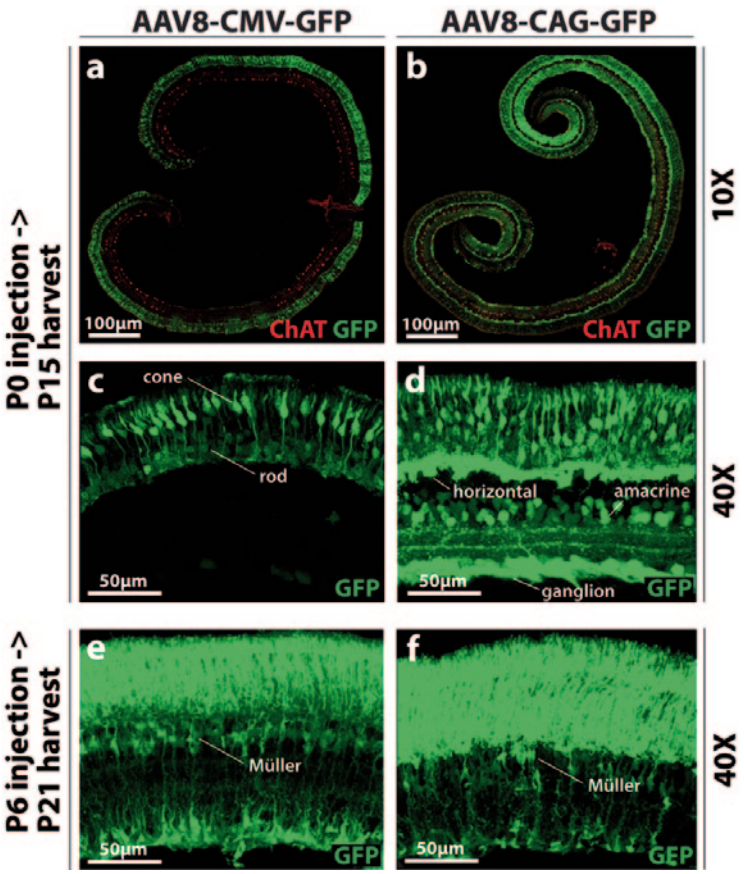

Fig 67.2 The entire cross sections of AAV8-CMV-GFP **(a)** and AAV8-CAG-GFP (**b**) P0 infected eyes. Retinal sections were imaged for native GFP signal (*green*) and Cy3 for ChAT staining (*red*), which highlighted laminae 2&4 in the INL. **c–d** Higher magnification images of the retinas shown in **a–b**. Representative cell types that were efficiently infected and expressed GFP are labeled. **e–f** Higher magnification images of retinal sections from P6 infected and P21 harvested retinas. In both groups of retinas, photoreceptors and Müller glia were efficiently transduced

diffuse across the retina to infect the innermost cells. The fact that no GFP signal was observed in these cell types following AAV8-CMV-GFP P0 subretinal infection must be a reflection of the regulatory elements in this vector. This is consistent with the previous reports that the CMV element has more variability in expression than other broadly active elements and that it is silenced in some cell types (Qin et al. 2010). Although the term "promoter" is often used to describe these elements, it is worth noting that not only the promoter sequence, but also the intron and splice sequences, differ among these vectors. In the CAG vector, the majority of the intron is from the chicken β-actin intron 1, which is thought to include enhancer activity (Niwa et al. 1991). In the CMV vector, the intron and splice sites were taken from human β-globin intron b region. Furthermore, the human CMV IE enhancer in the CAG vector is a short version (~360 bp) of the one used in the CMV vector

(~700 bp). Any or all of these differences might contribute to the different expression patterns noted between these two vectors.

In addition to the differences in the patterns seen using the two sets of regulatory sequences, the timing of the subretinal injection resulted in a fairly dramatic difference in the final expression patterns. One developmental difference that may, at least in part, be responsible for these observations concerns the access of the virions to the inner retina. P6 subretinal injections of either AAV8-CMV-GFP or AAV8-CAG-GFP resulted in efficient GFP expression in photoreceptors and Müller glial cells, but not in inner retinal neurons. Müller glia are born postnatally, and form the outer limiting membrane (OLM). Although the timing of formation of the OLM has not been specifically tracked in mice, it may be at least partially in place by P5 (Uga and Smelser 1973). The OLM may restrict the diffusion of AAV to the inner retina, but allow access of AAV to photoreceptors, which have their developing inner/outer segments protruding beyond the OLM. Similarly, Müller glia may be infected through their OLM processes. Higher titer inoculations, or viruses with other capsids, may produce more infection of the INL, but were not tested here.

AAV injection relative to a cell's last cell cycle may also be important in determining the expression pattern. Transgene expression may be different following introduction into mitotic vs. postmitotic cells. Given that AAV does not replicate, and the vector form integrates with a very low efficiency into the host cell's genome (McCarty et al. 2004), an AAV genome will be passed on to only one daughter cell in each cell cycle. It might be the case that an initially infected postmitotic cell retains many or all of the AAV genomes delivered by the inoculum, but the daughters of mitotic cells receive a diluted number of AAV genomes. This may explain why cones, horizontal, and retinal ganglion cells have the highest GFP expression level in AAV8-CAG-GFP P0 infected retinas, as these cells are postmitotic by P0. This is also in keeping with the curious finding that inner rods, relative to outer rods, express GFP more highly following infection at P0. Birthdating studies have shown that the inner rods are born before or at P0-P1, while the majority of the outer rods are born after P0 (Young 1985). Both inner and outer rods express GFP equally well following P6 infection, so there is no intrinsic difference between them regarding their use of the viral regulatory sequences. The fate of the AAV genome in mitotic may also differ from its fate in postmitotic cells. Silencing, or destruction of the genome, are additional possibilities for the lack of GFP expression in the descendants of infected mitotic cells.

One additional aspect of the expression patterns to be considered is the absence of expression in bipolar cells using either vector and infection time. This could be due to a lack of diffusion of virions to bipolar cells at P6, and/or a lack of a receptor on bipolar cells for AAV8, and/or lack of activity of the regulatory elements in bipolar cells. We have noted that CMV-based plasmids do not express as highly in bipolar cells as they do in Müller glia when plasmids are delivered by electroporation into retinal progenitor cells, suggesting a limitation in the strength of these elements in bipolar cells (Matsuda and Cepko 2004). Moreover, AAV8 vectors with a Grm6 promoter, which is active in ON-bipolar cells, can express in bipolar cells when delivered to adult murine retinas from intravitreal injections, when the capsid

has a tyrosine mutation in the capsid (Doroudchi et al. 2011). This mutation presumably reduces proteosomal degradation of the capsid in bipolar cells and thus may result in a higher copy number of AAV genomes in bipolar cells. Recent reports have shown improved expression of AAV-encoded genes in bipolar cells following intravitreal injection in adults. These vectors had changes in the capsid (Cronin et al. 2014; Macé et al. 2014) and improvements in the regulatory elements that were more active in bipolar cells (Cronin et al. 2014). These findings indicate that both infection and expression in bipolar cells need to be addressed for efficient expression, at least from intravitreal injections, and this may well be true for successful expression in bipolar cells following subretinal injections as well.

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