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The vesicular glutamate transporter-1 upstream promoter and first intron each support glutamatergic-specific expression in rat postrhinal cortex

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Abstract

Multiple applications of direct gene transfer into neurons require restricting expression to glutamatergic neurons, or specific subclasses of glutamatergic neurons. Thus, it is desirable to develop and analyze promoters that support glutamatergic-specific expression. The three vesicular glutamate transporters (VGLUTs) are found in different populations of neurons, and VGLUT1 is the predominant VGLUT in the neocortex, hippocampus, and cerebellar cortex. We previously reported on a plasmid (amplicon) Herpes Simplex Virus vector that contains a VGLUT1 promoter. This vector supports long-term expression in VGLUT1-containing glutamatergic neurons in rat postrhinal (POR) cortex, but does not support expression in VGLUT2-containing glutamatergic neurons in the ventral medial hypothalamus. This VGLUT1 promoter contains both the VGLUT1 upstream promoter and the VGLUT1 first intron. In this study, we begin to isolate and analyze the glutamatergic-specific regulatory elements in this VGLUT1 promoter. We show that the VGLUT1 upstream promoter and first intron each support glutamatergic-specific expression. We isolated a small, basal VGLUT1 promoter that does not support glutamatergic-specific expression. Next, we fused either the VGLUT1 upstream promoter or the first intron to this basal promoter. The VGLUT1 upstream promoter or the first intron, fused to the basal promoter, each supported glutamatergic-specific expression in POR cortex.

Keywords

glutamatergic neuron class; glutamatergic neuron-specific expression; vesicular glutamate transporter 1 promoter; neocortical neuron; herpes simplex virus vector

1. Introduction

Due to the numerous classes and subclasses of neurons in the brain, and especially in the forebrain, neuron class-specific expression is essential for many neural direct gene transfer studies or gene therapies. Glutamatergic neurons are the major class of excitatory neurons in the central nervous system, although the subclasses of glutamatergic neurons remain

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controversial (Nelson et al., 2006; Sugino et al., 2006). Thus, it is important to develop vectors that restrict expression to glutamatergic neurons, or specific subclasses of these neurons. One approach is to develop promoters that support expression in these classes of neurons.

Helper virus-free Herpes Simplex Virus (HSV-1) plasmid vectors (Fraefel et al., 1996) (amplicons) are advantageous for neuronal gene transfer, and particularly for achieving neuron class-specific expression. These vectors efficiently transduce neurons, they have a large capacity that can accommodate large cellular promoters (51 kb and 149 kb vectors are established (Wade-Martins et al., 2003; Wang et al., 2000)), and vectors containing specific cellular promoters support long-term, neuron class-specific expression: The tyrosine hydroxylase (TH) promoter supports expression in specific classes of catecholaminergic midbrain neurons (Jin et al., 1996; Song et al., 1997; Wang et al., 1999); the preproenkephalin (preproENK) promoter supports expression in specific brain areas that contain enkephalinergic neurons (Kaplitt et al., 1994a); and the glutamic acid decarboxylase (GAD) promoter supports GABAergic neuron-specific expression in neocortex, specifically in postrhinal (POR) cortex (Rasmussen et al., 2007). Also, neuron-specific expression in specific midbrain and forebrain areas is supported by chimeric promoters that contain an upstream enhancer from the TH promoter fused to the neurofilament heavy gene promoter (TH-NFH promoter), or add a β -globin insulator (INS) upstream of the TH-NFH promoter (INS-TH-NFH promoter) (Cao et al., 2008; Sun et al., 2004; Zhang et al., 2000; Zhang et al., 2005). HSV-1 vectors containing each of these promoters support long-term expression, for two to fourteen months (see references for each promoter, above).

HSV-1 vectors that support glutamatergic-specific expression have been obtained by using promoters for specific glutamate biosynthesis or synaptic vesicle transporter genes. GLS1 encodes the brain/kidney phosphate-activated glutaminase (PAG (Banner et al., 1988)), which produces most of the glutamate for release as neurotransmitter (Hertz, 2004). A vector containing the PAG promoter supports long-term (2 month), glutamatergic-specific expression in POR cortex (Rasmussen et al., 2007). Expression in specific subclasses of glutamatergic neurons has been achieved by using the vesicular glutamate transporter-1 (VGLUT1) promoter (Rasmussen et al., 2007; Zhang and Geller, 2010). The three VGLUTs are expressed in distinct populations of neurons (review (Freneau et al., 2004)). VGLUT1 is the predominant vesicular glutamate transporter in the neocortex, cerebellar cortex, hippocampus, and amygdala basolateral nuclei; VGLUT2 is the major one in the thalamus, deep cerebellar nuclei, hypothalamus, brainstem, and is also present in specific neocortical neurons, primarily in layer 4; and VGLUT3 is found in neurons traditionally viewed as non-glutamatergic (Bellocchio et al., 2000; Freneau et al., 2001; Freneau et al., 2004; Herzog et al., 2001; Takamori et al., 2000; Takamori et al., 2001; Varoqui et al., 2002). We previously showed that a HSV-1 vector containing the VGLUT1 promoter supports long-term (2 month) expression in VGLUT1-containing glutamatergic neurons in POR cortex, with minimal expression in VGLUT2-containing glutamatergic neurons in the ventromedial hypothalamus (Rasmussen et al., 2007; Zhang and Geller, 2010).

Importantly, particular neuron class-specific promoters contain multiple genetic elements that support expression in different subclasses of neurons. Studies using transgenic mice have shown that the TH, GAD65, and GAD67 promoters each contain multiple genetic elements that support expression in different subclasses of catecholaminergic (Liu et al., 1997) or GABAergic (Bali et al., 2005; Chattopadhyaya et al., 2004; Di Cristo et al., 2004; Heinke et al., 2004; Kobayashi et al., 2003; Lopez-Bendito et al., 2004; Ma et al., 2006; Makinae et al., 2000; Oliva et al., 2000; Oliva et al., 2002; Tamamaki et al., 2003) neurons, respectively.

Thus, we initiated an analysis of the VGLUT1 promoter. The VGLUT1 promoter we have examined contains both the upstream promoter and the first intron. In this study, we isolated a small, basal VGLUT1 promoter that does not support glutamatergic-specific expression. Next, we fused either the VGLUT1 upstream promoter or the first intron to this basal promoter. These two regulatory elements each supported glutamatergic-specific expression in POR cortex.

2. Results

2.1. A vector containing a 323 bp basal VGLUT1 promoter supports expression in both glutamatergic and GABAergic neurons

All the HSV-1 vectors in this study use our standard vector backbone (Fig. 1A (Song et al., 1997; Zhang et al., 2000)). pVGLUT1lac-Pr&Intron contains the mouse VGLUT1 upstream promoter and first intron (Fig. 1B; 7 kb and 4.6 kb fragments, respectively (Rasmussen et al., 2007)). In two previous reports, this vector was designated as pVGLUT1lac (Rasmussen et al., 2007; Zhang and Geller, 2010). The first intron may have a role in regulating expression (Dr. Herzog, personal communication) and thus was included in pVGLUT1lac-Pr&Intron.

Our strategy to identify the regulatory elements in the VGLUT1 upstream promoter and first intron that support glutamatergic-specific expression was to first isolate a basal VGLUT1 promoter that does not support glutamatergic-specific expression, and then fuse either a VGLUT1 upstream promoter or the first intron to the basal promoter, and evaluate the resulting promoters/regulatory elements for supporting glutamatergic-specific expression. To this end, we isolated a 323 bp basal promoter that contains the VGLUT1 transcription start site and extends in the 5' direction (upstream) for 241 bp (Fig. 1B). We inserted this basal promoter into the HSV-1 vector backbone to yield pVGLUT1lac-BasalPr.

pVGLUT1lac-Pr&Intron and pVGLUT1lac-BasalPr were packaged into HSV-1 particles, using a helper-virus free packaging system (Fraefel et al., 1996; Sun et al., 1999). The numbers of infectious vector particles (IVP/ml) were quantified by titrating the vector stocks on Baby Hamster Kidney (BHK) cells; at 1 day after transduction, positive cells were visualized using 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal) staining. The titers of the pVGLUT1lac-Pr&Intron and pVGLUT1lac-BasalPr vector stocks were similar (Table 1). {pVGLUT1lac-Pr&Intron has been characterized in two previous studies, designated Exp. 1 (Rasmussen et al., 2007) and Exp. 2 (Zhang and Geller, 2010) in Table 1. Exp. 2 was performed in parallel with the experiments in this study, and the previously published results from pVGLUT1lac-Pr&Intron Exp. 2 (Zhang and Geller, 2010) serve as a positive control in this study. All the data on the other vectors in this study are new and have not been previously published.} This titrating was performed on BHK fibroblast cells as the best available assay; these fibroblast cells form a monolayer. In contrast, most neuronal cell lines, such as PC12 cells, do not form a monolayer, and the titers obtained on BHK cells are higher than the titers obtained on specific neuronal cell lines (Yang et al., 2001; Zhang et al., 2000). Expression from specific VGLUT1 promoters/regulatory elements in fibroblast cells represents ectopic expression that declined rapidly at longer times after gene transfer (not shown). We used a previously established PCR assay (Yang et al., 2001) to determine the titer of vector genomes (VG/ml). The packaging efficiencies (VG/ml / IVP/ml) for pVGLUT1lac-Pr&Intron and pVGLUT1lac-BasalPr were calculated from the titers (Table 1). The packaging efficiency for pVGLUT1lac-Pr&Intron Exp. 1 (Rasmussen et al., 2007) and pVGLUT1lac-Pr&Intron Exp. 2 (determined in this study) were similar, and were similar to those observed for a number of other vectors we have studied (Rasmussen et al., 2007; Yang et al., 2001). pVGLUT1lac-BasalPr showed a >8-fold higher ratio of VG to IVP than pVGLUT1lac-Pr&Intron (see discussion).

We injected equal biological titers (IVP) of pVGLUT1lac-Pr&Intron or pVGLUT1lac-BasalPr, or 6.6-fold more physical particles (VG) of pVGLUT1lac-BasalPr than pVGLUT1lac-Pr&Intron (Table 1), into POR cortex, sacrificed the rats 4 days later, and performed costaining for β -galactosidase immunoreactivity (β -gal-IR) and PAG-IR, a marker for glutamatergic neocortical neurons (Kaneko and Mizuno, 1988; Kaneko et al., 1992; Sakata et al., 2002; Van der Gucht et al., 2003). Representative photomicrographs confirmed that pVGLUT1lac-Pr&Intron supports expression predominately in glutamatergic neurons (Fig. 2A–C), as was previously shown using this assay (Rasmussen et al., 2007; Zhang and Geller, 2010). Omitting the primary antibodies from the assay resulted in no detectable staining (Fig. 2D–F). In contrast, pVGLUT1lac-BasalPr supported expression in both glutamatergic and GABAergic neurons: Costaining for β -gal and PAG showed expression in many glutamatergic neurons (Fig. 3A–C); and costaining for β -gal and GAD, a marker for GABAergic neurons, showed expression in many GABAergic neurons (Fig. 3D–F).

For each vector, we performed cell counts to quantify the percentage of expressing cells that were glutamatergic or GABAergic neurons. The results from two previous studies (Rasmussen et al., 2007; Zhang and Geller, 2010) showed that pVGLUT1lac-Pr&Intron supports >90 % glutamatergic neuron-specific expression, and 13 % inappropriate expression in GABAergic neurons (Table 2; pVGLUT1lac-Pr&Intron Exp 2 was performed in parallel with the present study, as noted above). In contrast, pVGLUT1lac-BasalPr supports expression in both glutamatergic and GABAergic neurons, specifically 53 % glutamatergic-specific expression and 48 % GABAergic-specific expression. Similarly, a vector containing a neuron-specific promoter, a modified neurofilament heavy gene promoter (INS-TH-NFH promoter), supports 52 % glutamatergic-specific expression and 45 % GABAergic-specific expression in POR cortex (Zhang et al., 2005).

2.2. Fusing either a VGLUT1 upstream promoter or the VGLUT1 first intron to the basal promoter results in glutamatergic-specific expression

Following our strategy to identify glutamatergic-specific genetic elements, we fused either a VGLUT1 upstream promoter or the VGLUT1 first intron to the VGLUT1 basal promoter, and inserted the resulting promoters/regulatory elements into our HSV-1 vector backbone (Fig. 1). pVGLUT1lac-Pr&Intron contains a 7 kb promoter fragment, which can be viewed as a fusion of a 6.7 kb VGLUT1 upstream promoter to the 0.3 kb basal promoter. Thus, we inserted this 7 kb VGLUT1 promoter into a HSV-1 vector (Fig. 1B, pVGLUT1lac-Pr). {pVGLUT1lac-Pr was previously isolated as an intermediate in constructing pVGLUT1lac-Pr&Intron (Rasmussen et al., 2007), but pVGLUT1lac-Pr was not packaged into HSV-1 particles or tested in the brain, before this present study.} pVGLUT1lac-Pr&Intron contains the first intron, and this 4.6 kb fragment was fused to the 0.3 kb VGLUT1 basal promoter to yield pVGLUT1lac-BasalPr&Intron (Fig. 1B). pVGLUT1lac-Pr and pVGLUT1lac-BasalPr&Intron were packaged into HSV-1 particles, and the resulting vector stocks were titrated for both IVP/ml and VG/ml (Table 1). The results showed that both vectors were efficiently packaged into HSV-1 particles with titers, and packaging efficiencies, similar to those obtained using pVGLUT1lac-Pr&Intron.

We injected equal biological titers (IVP) of pVGLUT1lac-Pr or pVGLUT1lac-BasalPr&Intron, or 2.3- fold more physical particles (VG) of pVGLUT1lac-BasalPr&Intron than pVGLUT1lac-Pr (Table 1), into POR cortex, sacrificed the rats 4 days later, and determined the classes of neurons that support expression by costaining for β -gal-IR and either PAG-IR or GAD-IR. The results showed that pVGLUT1lac-Pr supported predominately glutamatergic-specific expression (Fig. 4A–C), with a low level of inappropriate expression in GABAergic neurons (Fig. 4D–F). Moreover, the results showed that pVGLUT1lac-BasalPr&Intron also supported predominately glutamatergic-specific

expression (Fig. 5A–C), with expression in small numbers of GABAergic neurons (Fig. 5D–F).

The expressing cells were scored for containing glutamatergic or GABAergic neuron markers, and the results showed that both pVGLUT1lac-Pr and pVGLUT1lac-BasalPr&Intron supported glutamatergic-specific expression (Table 2). Specifically, pVGLUT1lac-Pr supported 82 % glutamatergic-specific expression and 19 % GABAergic-specific expression. Additionally, pVGLUT1lac-BasalPr&Intron supported 88 % glutamatergic-specific expression and 17 % GABAergic-specific expression. We performed statistical tests to search for significant differences in the levels of glutamatergic or GABAergic-specific expression supported by these vectors. Two-way ANOVAs showed a significant effect of neuron class ($p < 0.001$); however, there was no significant effect of vector ($p > 0.05$), but there was a significant interaction between vector and neuron class ($p < 0.001$). Importantly, pairwise comparisons (Student-Neuman-Keuls test) on vector within the glutamatergic neuron class showed that each vector supported different levels of glutamatergic-specific expression than the other three vectors (all $p < 0.04$). In contrast to the results on glutamatergic-specific expression, pairwise comparisons on vector within the GABAergic neuron class showed that pVGLUT1lac-BasalPr supported higher levels of GABAergic-specific expression than the other three vectors ($p < 0.001$), but the three other vectors supported similar levels of GABAergic-specific expression (all $p > 0.05$). Of note, some modest errors in the assays for glutamatergic or GABAergic-specific expression should be considered in evaluating the results of these statistical tests: pVGLUT1lac-Pr&Intron Exp. 1 showed 92 % glutamatergic-specific expression and 13 % GABAergic-specific expression, for a total of 105 % (Table 2; 92 % + 13 % = 105 %). Similarly, pVGLUT1lac-BasalPr, pVGLUT1lac-Pr, and pVGLUT1lac-BasalPr&Intron showed totals of 101, 101, and 105 %, respectively (Table 2: pVGLUT1lac-BasalPr, 53 % glutamatergic and 48 % GABAergic (53 % + 48 % = 101 %); pVGLUT1lac-Pr, 82 % glutamatergic and 19 % GABAergic (82 % + 19 % = 101 %); pVGLUT1lac-BasalPr&Intron, 88 % glutamatergic and 17 % GABAergic (88 % + 17 % = 105 %)). Inappropriate expression in specific classes of glia was not assayed in this study. In summary, pVGLUT1lac-BasalPr supports expression in both glutamatergic and GABAergic neurons; in contrast, pVGLUT1lac-Pr&Intron, pVGLUT1lac-Pr, and pVGLUT1lac-BasalPr&Intron each support glutamatergic-specific expression, and these three vectors may support different levels of glutamatergic-specific expression (see discussion).

2.3. The numbers of expressing cells supported by pVGLUT1lac-Pr, pVGLUT1lac-BasalPr&Intron, and controls

We determined the total number of expressing cells per hemisphere (injection site) supported by each vector (Table 3). pVGLUT1lac-Pr&Intron Exp. 1 had a higher titer (IVP/ml) than the other vector stocks used in this study (Table 1), and the number of transduced cells does not increase linearly with the injected titer; further, for pVGLUT1lac-Pr&Intron Exp. 1, expression was assayed using X-gal, which is modestly less sensitive than the β -gal-IR assay used for the other vector stocks. Thus, the number of expressing cells supported by pVGLUT1lac-Pr&Intron Exp. 1 cannot be meaningfully compared to that supported by the other four vector stocks, and the pVGLUT1lac-Pr&Intron Exp. 1 data was included in Table 3 for completeness. The biological titers (IVP/ml) of the vector stocks for pVGLUT1lac-Pr&Intron Exp. 2, pVGLUT1lac-BasalPr, pVGLUT1lac-Pr, and pVGLUT1lac-BasalPr&Intron were similar (Table 1; 1.8 to 2.0×10^6 IVP/ml), the physical titers for the three vectors that support glutamatergic-specific expression were within a 2.7-fold range (Table 1; pVGLUT1lac-Pr&Intron Exp. 2, pVGLUT1lac-Pr, and pVGLUT1lac-BasalPr&Intron 2.9 to 7.8×10^7 VG/ml), and the total numbers of expressing cells in POR cortex was determined by the same assay for each vector stock, β -gal-IR. Thus, the total

numbers of expressing cells supported by these four vector stocks were compared. Of note, pVGLUT1lac-Pr and pVGLUT1lac-BasalPr&Intron each supported expression in <50 % of the number cells per hemisphere as pVGLUT1lac-Pr&Intron Exp. 2 (Table 3). These differences were statistically significant ($p < 0.001$ one-way ANOVA. Pairwise comparisons: pVGLUT1lac-Pr or pVGLUT1lac-BasalPr&Intron vs. pVGLUT1lac-Pr&Intron $p < 0.001$; pVGLUT1lac-Pr vs. pVGLUT1lac-BasalPr&Intron $p > 0.05$). Also, pVGLUT1lac-BasalPr supported expression in a similar number of cells as pVGLUT1lac-Pr&Intron (pVGLUT1lac-BasalPr vs. pVGLUT1lac-Pr&Intron $p > 0.05$; pVGLUT1lac-BasalPr vs. either pVGLUT1lac-Pr or pVGLUT1lac-BasalPr&Intron $p < 0.001$). Interestingly, pVGLUT1lac-Pr and pVGLUT1lac-BasalPr&Intron together supported expression in an average of 431 cells per hemisphere (Table 3; $204 + 227 = 431$); similarly, pVGLUT1lac-Pr&Intron supported expression in an average of 544 cells per hemisphere. These results raise the possibility that pVGLUT1lac-Pr and pVGLUT1lac-BasalPr&Intron may support expression in specific, and predominately different, subclasses of glutamatergic neurons; however, several issues complicate analysis of these cell counts (see discussion).

3. Discussion

Here, we establish that the VGLUT1 upstream promoter or the first intron, after fusion to a basal promoter, each support glutamatergic-specific expression. A small, ~300 bp VGLUT1 basal promoter supports expression in approximately equal numbers of glutamatergic and GABAergic neurons. In contrast, an upstream VGLUT1 promoter or the first intron, fused to the basal promoter, each support glutamatergic-specific expression.

Similar to a number of other neuron-specific promoters, a small ~300 bp VGLUT1 basal promoter supports expression in both glutamatergic and GABAergic neurons. Of note, a vector containing a modified neurofilament heavy gene promoter (INS-TH-NFH promoter), supports expression in approximately equal numbers of glutamatergic and GABAergic neurons in POR cortex (Zhang et al., 2005). Moreover, an ~650 bp NFH promoter supports neuron-specific expression (Schwartz et al., 1994; Wang et al., 1999; Zhang et al., 2000), and an ~800 bp TH promoter does not support catecholaminergic specific-expression, but instead supports neuron-specific expression, in both transgenic mice and HSV-1 vectors (Liu et al., 1997; Wang et al., 1999). {In contrast, large 6.0 or 9 kb fragments of the TH promoter are required for catecholaminergic specific-expression in transgenic mice (Liu et al., 1997; Min et al., 1994).} Thus, small promoters; such as ~300 to ~800 bp fragments of the VGLUT1, NFH, and TH promoters; can support neuron-specific expression.

The glutamatergic-specific expression observed here is due to the properties of specific VGLUT1 promoters/regulatory elements and not preferential transduction of glutamatergic neurons by this HSV-1 vector system. HSV-1 vectors containing either a modified neurofilament heavy gene promoter (INS-TH-NFH) or the VGLUT1 basal promoter each support expression in similar numbers of GABAergic and glutamatergic neurons in POR cortex ((Zhang et al., 2005) or this study, respectively). Further, HSV-1 vectors containing the GAD, PAG, or the VGLUT1 promoters support expression predominately in either GABAergic or glutamatergic neurons, respectively, in POR cortex ((Rasmussen et al., 2007; Zhang and Geller, 2010) and this study). Thus, this vector system transduces similar numbers of GABAergic and glutamatergic neurons in POR cortex, and neuron class-specific expression is due to the properties of specific promoters.

We quantified neuron class-specific recombinant expression by a standard approach, costaining for a recombinant protein and markers for specific types of neurons. Similarly, in studies in the rodent midbrain; using lentivirus, adenovirus, or HSV-1 vectors; catecholaminergic-specific expression was quantified by costaining for a recombinant

protein and TH-IR (Carlezon et al., 2000; Choi-Lundberg et al., 1997; Georgievska et al., 2004; Jin et al., 1996; Romano et al., 2005; Song et al., 1997). Further, in studies in the rat or monkey striatum; using adenovirus, lentivirus, adeno-associated virus (AAV), or HSV-1 vectors; the cell classes supporting recombinant expression were determined by costaining for a recombinant protein and NeuN, neurofilament, MAP2, GAD, DARP32, or GFAP (Azzouz et al., 2002; Corti et al., 1999; Kaplitt et al., 1994b; Mandel et al., 1998; Naldini et al., 1996; Shen et al., 2000; Sun et al., 2004). Moreover, in studies in the rodent hippocampus; using lentivirus, adenovirus, AAV, or HSV-1 vectors; a recombinant protein was localized to neurons by costaining for NeuN (Blomer et al., 1997; Naldini et al., 1996; Zhang et al., 2009). Further, numerous studies using transgenic mice have performed costaining for a recombinant protein and a marker for neurons, or a specific class of neuron. Thus, our assays for glutamatergic-specific or GABAergic-specific expression, by costaining for β -gal-IR and either PAG-IR or GAD-IR, respectively, use a standard approach.

Each of the three large VGLUT1 promoters/regulatory elements studied here supported a low level of inappropriate expression in GABAergic neurons. Some of the inappropriate expression could be because the chromatin structure on the vector has not reached steady state levels by 4 days after gene transfer. However, similar low levels of inappropriate expression were also observed at both short and long times after gene transfer, using HSV-1 vectors containing particular neuron-specific, or neuron class-specific, promoters, including a modified neurofilament heavy gene promoter (INS-TH-NFH), preproENK, TH, PAG, and GAD promoters (Jin et al., 1996; Kaplitt et al., 1994a; Rasmussen et al., 2007; Song et al., 1997; Sun et al., 2003; Sun et al., 2004; Sun et al., 2005; Wang et al., 1999; Zhang et al., 2000). This inappropriate expression could be due to specific missing promoter/regulatory elements, missing developmental gene regulatory events because gene transfer is into young adult rats, side effects from the vector system, or other causes. This helper virus-free vector system causes minimal side-effects; nonetheless, specific side effects can cause inappropriate expression: For example, a vector containing a TH promoter supported 40 % catecholaminergic-specific expression in a helper virus-containing system (Song et al., 1997), but ~61 % specific expression in the helper virus-free system used here (Wang et al., 1999); and this helper virus-free system causes significantly less cytotoxicity and immune response than helper virus-containing systems (Fraefel et al., 1996; Olschowka et al., 2003). Alternatively, although the entire VGLUT1 promoter is a large 11.6 kb fragment (Rasmussen et al., 2007), specific essential promoter/regulatory elements may be missing. In transgenic mice, bacterial artificial chromosomes (BACs), which can be over 100 kb, can support a higher level of cell class-specific expression than smaller fragments. Another possibility is that the inappropriate expression could be due to genetic regulatory events that occur during the differentiation of glutamatergic neurons; these regulatory events may not be reproduced when HSV-1 vectors transduce mature glutamatergic neurons in young adult rats. For example, as a hypothetical step in the activation of the VGLUT1 promoter during differentiation, a specific histone on the VGLUT1 promoter may be modified (phosphorylation, acetylation, methylation, etc.), or a specific sequence in the VGLUT1 promoter DNA may be modified (methylation, etc), and this event may not occur after transduction of mature glutamatergic neurons. Another possibility is that the mouse VGLUT1 promoter was studied in rats, and, although these species are closely related, difference in the sequences of the VGLUT1 promoters between these species could cause a low level of inappropriate expression. However, human and rat TH promoters, or rat synapsin I or rat neuron-specific enolase promoters, support catecholaminergic-specific expression (Liu et al., 1997; Min et al., 1994; Sasaoka et al., 1992) or neuron-specific expression (Forss-Petter et al., 1990; Hoesche et al., 1993), respectively, in transgenic mice.

Each of the three large VGLUT1 promoters/regulatory elements studied here supported high levels of glutamatergic-specific expression. Some of the results suggest that the entire VGLUT1 promoter supports higher levels of glutamatergic-specific expression than either the upstream promoter or the first intron, alone: The entire VGLUT1 promoter supported a statistically significant higher level of glutamatergic-specific expression than the first intron, which supported a higher level of glutamatergic-specific expression than the upstream promoter. The glutamatergic-specific element(s) in the upstream promoter or the first intron may be enhancers because they act at a distance (these are large fragments); however, we did not examine orientation-independence. In contrast, these three VGLUT1 promoters/regulatory elements supported statistically similar levels of inappropriate expression in GABAergic neurons, likely the major cell class for inappropriate expression. Further, the total percent expression (% glutamatergic-specific expression plus % GABAergic-specific expression) for each of these three promoters/regulatory elements was close to, but not precisely, 100 %, documenting some inherent error in these assays. Moreover, the potential sources of inappropriate expression detailed in the previous paragraph apply to the issue under discussion here. Nonetheless, it is possible that the upstream promoter and the first intron fragments each contain specific genetic elements that block inappropriate expression in particular classes of non-glutamatergic cells; in this case, the entire VGLUT1 promoter would support a higher level of glutamatergic-specific expression than either fragment alone. Deletion analyses of the two fragments defined here might define the genetic regulatory elements in each fragment.

The ratios of physical to biological titers for the vectors containing the three large VGLUT1 promoters/regulatory elements were similar (Table 1), these ratios were similar to those observed with other specific vectors, and were different from that supported by the vector containing the VGLUT1 basal promoter. Similar packaging efficiencies were supported by vectors containing the HSV-1 immediate early 4/5 promoter (Wang et al., 2005), specific modified neurofilament promoters (INS-TH-NFH, TH-NFH, or specific deletions of the TH fragment in TH-NFH (Gao et al., 2006; Gao et al., 2007; Liu et al., 2005; Liu et al., 2009; Yang et al., 2001)), the PAG or GAD promoters (Rasmussen et al., 2007), or each of the three large VGLUT1 promoters/regulatory elements ((Rasmussen et al., 2007) and this study); these ratios of VG/ml to IVP/ml ranged from 1 to 45. These promoters were studied because they support high-level expression in the brain; other specific promoters support lower levels of expression (Wang et al., 1999), and their packaging efficiencies were not determined. In contrast to the vectors/promoters just listed, the vector containing the basal VGLUT1 promoter supported a >8-fold higher ratio of VG/ml to IVP/ml than the vector containing the entire VGLUT1 promoter. This difference is not a sequence-specific effect on the packaging efficiency because each of the three large VGLUT1 promoters/regulatory elements contain the entire basal VGLUT1 promoter, and these three vectors were each packaged as efficiently as a number of other vectors containing other specific promoters (references cited above). Rather, the higher ratio of VG/ml to IVP/ml for the basal promoter vector suggests that the basal promoter is a modestly weaker promoter than the other promoters in these comparisons. Consistent with interpretation, injection of equal biological titers (IVP) of vectors containing either the INS-TH-NFH promoter or the entire VGLUT1 promoter results in ~2-fold more expressing cells from the vector containing the INS-TH-NFH promoter (Zhang and Geller, 2010), because the INS-TH-NFH promoter is active in both GABAergic and glutamatergic neurons but the VGLUT1 promoter is active only in glutamatergic neurons. In contrast, injection of equal biological titers of vectors containing either the basal VGLUT1 promoter or the entire VGLUT1 promoter resulted in similar numbers of expressing cells (Table 3), although the basal VGLUT1 promoter is active in both GABAergic and glutamatergic neurons. Of note, addition of either the VGLUT1 upstream promoter or the first intron to the basal promoter results in packaging efficiencies that are similar to those for all the other vectors/promoters discussed here. Thus, the

strengths of the three large VGLUT1 promoters/regulatory elements are similar, at least at the level of a β -gal-positive-cell assay in fibroblast cells, which does not detect modest differences in promoter strength.

The data indirectly suggest that the VGLUT1 upstream promoter or first intron, fused to the basal promoter, may support expression in specific, and possibly predominately different, subclasses of glutamatergic neurons. The upstream promoter or the first intron each supported expression in statistically fewer cells than the entire VGLUT1 promoter, suggesting that each of these elements supports expression in specific subclasses of glutamatergic neurons. Of note, the upstream promoter and the first intron together represent the entire VGLUT1 promoter. Thus, if these two vectors support expression in predominately different subclasses of glutamatergic neurons, then the sum of the number of expressing cells supported by these two vectors would approximately equal the number of expressing cells supported by the vector containing the entire promoter. The results (Table 3) support this possibility. In contrast, if the upstream promoter and the first intron support expression in some of the same subclasses of glutamatergic neurons, then the sum of the number of expressing cells supported by these two vectors would exceed the number of expressing cells supported by the vector containing the entire promoter. This was not observed. However, interpretation of the cell counts is complicated by several issues. As detailed above, the three vectors containing the large VGLUT1 promoters/regulatory elements exhibit similar physical titers, packaging efficiencies, and similar promoter strengths at the level of a β -gal-positive-cell assay, which used fibroblast cells. However, any differences in promoter strength between these three vectors in glutamatergic neurons are not known. Also, any potential interactions between elements in the upstream promoter and first intron can only occur in the vector containing the full promoter, and these could affect relative promoter strengths. Moreover, a limited number of injections of each vector were performed, and different layers of POR cortex contain different densities of glutamatergic neurons, which could affect the numbers of expressing cells. Thus, further experiments, beyond the scope of this study, are now indicated to directly determine if the upstream promoter and the first intron support expression in specific, and predominately different, subclasses of glutamatergic neurons. Nonetheless, the TH, GAD65, and GAD67 promoters each contain multiple genetic elements that support expression in different subclasses of catecholaminergic (Liu et al., 1997) or GABAergic (Bali et al., 2005; Chattopadhyaya et al., 2004; Di Cristo et al., 2004; Heinke et al., 2004; Kobayashi et al., 2003; Lopez-Bendito et al., 2004; Ma et al., 2006; Makinae et al., 2000; Oliva et al., 2000; Oliva et al., 2002; Tamamaki et al., 2003) neurons, respectively.

4. Materials and Methods

4.1. Materials

OptiMEM, penicillin/streptomycin, Dulbecco's modified minimal essential medium (DMEM), and fetal bovine serum (FBS) were obtained from Invitrogen; G418 was obtained from RPI; and X-gal was from Sigma. Mouse anti-*E. coli* β -gal was obtained from Sigma; rabbit anti-PAG was a generous gift from Dr. N. Curthoys, and rabbit anti-GAD was obtained from Millipore. Texas red-conjugated horse anti-mouse immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were from Vector Labs.

4.2. Vectors

All the vectors in this study used our standard vector backbone (Fig. 1A; (Song et al., 1997; Zhang et al., 2000)). The promoters/regulatory elements in the vectors are diagrammed in Fig. 1B. pVGLUT1lac-Pr&Intron has been previously described and contains the mouse

VGLUT1 upstream promoter and first intron (7 kb and 4.6 kb fragments, respectively (Rasmussen et al., 2007)); this vector was designated as pVGLUT1lac in previous publications (Rasmussen et al., 2007; Zhang and Geller, 2010). The construction of pVGLUT1lac-Pr was previously described (this vector was designated as pVGLUT1-prom-lac in (Rasmussen et al., 2007)), and this vector contains the VGLUT1 upstream promoter (7 kb fragment), but lacks the first intron.

To construct pVGLUT1lac-BasalPr, we first isolated a 323 bp fragment that contains the VGLUT1 promoter transcription start site and extends in the 5' direction (upstream) for 241 bp. PCR was performed using pVGLUT1lac-Pr&Intron as template and primers (sense 5' ACCACTTAAGTCGGAGTTCAATCCCAGGACGTGGC 3' and antisense 5' ACCAGCGGCCGCGTCCGGGATCCCGGGGTCCGCCCCGCCCCGGCCC 3'). The PCR products were cloned into the TOPO vector. The 323 bp basal promoter fragment was excised from the TOPO vector using Not I and Afl II, and inserted into pVGLUT1lac-Pr that had been digested with the same enzymes, to yield pVGLUT1lac-BasalPr.

To construct pVGLUT1lac-BasalPr&Intron, pVGLUT1lac-BasalPr was digested with Not I and Spe I, and the 0.35 kb fragment containing the VGLUT1 basal promoter was isolated. pVGLUT1lac-Pr&Intron was digested with the same enzymes, and the 13.8 kb fragment containing the vector backbone, VGLUT1 first intron, and Lac Z gene was isolated. These two fragments were ligated together to yield pVGLUT1lac-BasalPr&Intron.

4.3. Cells and vector packaging

BHK21 and 2-2 cells were maintained in DMEM supplemented with 10 % FBS, 4 mM glutamine, and penicillin/streptomycin, and they were grown in an incubator at 37 °C, 5 % CO₂, and 100 % humidity. G418 (0.5 mg/ml), present during the growth of 2-2 cells, was removed before plating cells for vector packaging.

Vectors were packaged into HSV-1 particles using a modified form of the helper-virus free packaging protocol described previously (Fraefel et al., 1996; Sun et al., 1999). Purified vectors were titrated for biological activity (IVP/ml) on BHK cells, by counting X-gal positive cells at 24 hrs after transduction. Packaged vector DNA (VG/ml) was quantified using a PCR assay for Lac Z sequences, as described (Yang et al., 2001). The titers of the vector stocks used in this study are in Table 1. Wild-type HSV-1 was not detected (<10 plaque forming units/ml) in any of the vector stocks.

4.4. Stereotactic injections of vectors into rat POR cortex

The VA Boston Healthcare System IACUC approved all the animal procedures. Adult male Long-Evans rats (150–200 g) were anesthetized by ip injection of a mixture of Ketamine (20 mg/ml) and Xylazine (2 mg/ml) with a final dose of 60 mg/kg and 6 mg/kg, respectively. Additional anesthesia was administered as needed. Each rat received 2 injections of a specific vector into POR cortex, one in each hemisphere: The injection coordinates were anterior-posterior (AP) –8.0, medial-lateral (ML) ±6.0, dorsal-ventral (DV) –5.2 (Paxinos and Watson, 1986). AP is measured relative to bregma, ML is relative to the sagittal suture, and DV is relative to the bregma-lambda plane. A micropump (model 100, KD Scientific) was used for the injections; 3 µl of vector stock was injected at each site over 5 minutes, and after 5 additional minutes, the needle was slowly retracted.

4.5. Immunohistochemistry

Brains were perfused as described (Zhang et al., 2000), and 25 µm coronal sections containing, or proximal to, the injection site were prepared using a freezing microtome. Rabbit anti-PAG antibody was purified using a prepacked diethylaminoethyl/Affi-Gel blue

column, following the manufacturer's instructions. Immunohistochemistry was performed on free-floating sections as described (Zhang et al., 2000). β -gal-IR was detected using mouse anti- β -gal antibody (1:500 dilution), and neuron classes were identified using either rabbit anti-PAG (1:400 dilution) or rabbit anti-GAD (1:500 dilution). Primary antibodies were visualized with Texas red-conjugated horse anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (1:200 dilutions).

4.6. Cell counts

Glutamatergic or GABAergic-specific expression was quantified by cell counts. Digital images were taken at 60X magnification, merged, and counts were performed on the merged images. In the fields that were examined, all the β -gal-IR cells were scored for containing, or lacking, a marker for a specific neuron class. All counts were done at least two separate times, and results differed by <10 %.

4.7. Statistical analyses

Descriptive statistics were calculated using Excel. Statistical tests were performed using AVOVAs (Sigmastat, SPSS Inc.).

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Fig. 1.

Schematic diagrams of the HSV-1 vector backbone and the four VGLUT1 promoters/regulatory elements that were inserted into this vector backbone. (A) Schematic diagram of the HSV-1 vector backbone (Song et al., 1997; Zhang et al., 2000). The HSV-1 *ori_S* and the HSV-1 *a* sequence (contains the DNA cleavage/packaging signals) support replication and packaging of vectors into HSV-1 particles. The tri-A cassette contains three SV40 early region polyadenylation sites to block any transcription from the HSV-1 *ori_S* fragment (contains the HSV-1 immediate early 4/5 promoter). Specific promoters were fused to the *Lac Z* gene, which is followed by the α -globin second intron and the SV40 early region polyadenylation site. The *E. coli amp^r* gene and the col E1 origin of DNA replication enable propagation in *E. coli*. (B) Schematic diagram of the four VGLUT1 promoters/regulatory elements. pVGLUT1lac-Pr&Intron contains a 7 kb upstream promoter fragment, which includes the transcription start site, and a 4.6 kb fragment that contains the first intron. pVGLUT1lac-BasalPr contains a 323 bp fragment that contains the transcription start site

and extends in the 5' direction (upstream) for 241 bp. pVGLUT1lac-BasalPr contains the 7 kb upstream promoter fragment, which includes the transcription start site. pVGLUT1lac-BasalPr&Intron contains the 323 bp fragment, containing the basal promoter and the transcription start site, fused to the 4.6 kb fragment that contains the first intron.

Fig. 2. pVGLUT1lac-Pr&Intron supported expression of β -gal predominantly in glutamatergic neurons in POR cortex. The rats were sacrificed at 4 days after gene transfer. β -gal-IR was detected using an anti- β -gal antibody, and glutamatergic neurons were identified by costaining using anti-PAG. (A–C) Most of the β -gal expressing cells were glutamatergic neurons; (A) β -gal-IR, (B) PAG-IR, or (C) merged. Arrows, costained cells; arrowheads, singly stained cells. (D–F) Omission of the primary antibodies resulted in no detectable immunofluorescence; (D) Texas red filter, (E) fluorescein filter, or (F) merged. Scale bar: 50 μ m.

Fig. 3. pVGLUT1lac-BasalPr supported expression of β -gal in both in glutamatergic and GABAergic neurons in POR cortex. The rats were sacrificed at 4 days after gene transfer. β -gal-IR was detected using anti- β -gal, and glutamatergic or GABAergic neurons in the same sections were identified by costaining using either anti-PAG or anti-GAD, respectively. (A–C) About half of the β -gal expressing cells were glutamatergic neurons; (A) β -gal-IR, (B) PAG-IR, or (C) merged. Arrows, costained cells; arrowheads, singly stained cells. (D–F) About half of the β -gal expressing cells were GABAergic neurons; (D) β -gal-IR, (E) GAD-IR, or (F) merged. Scale bar: 50 μ m.

Fig. 4.

pVGLUT1lac-Pr supported expression of β -gal predominantly in glutamatergic neurons in POR cortex. The rats were sacrificed at 4 days after gene transfer. β -gal-IR was detected using anti- β -gal, and glutamatergic or GABAergic neurons were identified by using either anti-PAG or anti-GAD, respectively. (A–C) Most of the β -gal expressing cells were glutamatergic neurons; (A) β -gal-IR, (B) PAG-IR, or (C) merged. Arrows, costained cells; arrowheads, singly stained cells. (D–F) Only a small fraction of the β -gal expressing cells were GABAergic neurons; (D) β -gal-IR, (E) GAD-IR, or (F) merged. Scale bar: 50 μ m.

Fig. 5. pVGLUT1lac-BasalPr&Intron supported expression of β -gal predominantly in glutamatergic neurons. The rats were sacrificed at 4 days after gene transfer. β -gal-IR was detected using anti- β -gal, and glutamatergic or GABAergic neurons were visualized using anti-PAG or anti-GAD, respectively. (A–C) Most of the β -gal expressing cells were glutamatergic neurons; (A) β -gal-IR, (B) PAG-IR, or (C) merged. Arrows, costained cells; arrowheads, singly stained cells. (D–F) A small fraction of the β -gal expressing cells were GABAergic neurons; (D) β -gal-IR, (E) GAD-IR, or (F) merged. Scale bar: 50 μ m.

Table 1

Titers of the vector stocks

Vector	IVP / ml	VG / ml	VG / IVP
^a pVGLUT1lac-Pr&Intron Exp. 1	6.5×10^6	9.6×10^7	15
^a pVGLUT1lac-Pr&Intron Exp. 2	2.0×10^6	2.9×10^7	15
pVGLUT1lac-BasalPr	1.8×10^6	1.9×10^8	127
pVGLUT1lac-Pr	2.0×10^6	3.4×10^7	17
pVGLUT1lac-BasalPr&Intron	2.0×10^6	7.8×10^7	39

After helper-virus free packaging (Fraefel et al., 1996; Sun et al., 1999), vector stocks were titered for biological activity on BHK cells; at 1 day after transduction, positive cells were visualized using X-gal, and the IVP/ml were determined. To quantify the VG/ml, DNA was isolated from the same vector stocks, and Lac Z sequences were detected by PCR as previously described (Yang et al., 2001).

^a pVGLUT1lac-Pr&Intron Exp. 1 IVP/ml and VG/ml, and pVGLUT1lac-Pr&Intron Exp. 2 IVP/ml were previously published (Rasmussen et al., 2007; Zhang and Geller, 2010). pVGLUT1lac-Pr&Intron Exp. 2 was packaged and titered in parallel with the other vectors in this study. pVGLUT1lac-Pr&Intron Exp. 1 was packaged and titered prior to this study, using the same methods.

Table 2

The levels of glutamatergic- and GABAergic-specific expression supported by vectors containing specific VGLUT1 promoters/regulatory elements

Vector	Glutamatergic-specific expression		GABAergic-specific expression	
	Average β -gal-IR cells	% PAG-IR costaining	Average β -gal-IR cells	% GAD-IR costaining
^a pVGLUT1lac-Pr&Intron Exp. 1	157±57	92±2	106±50	13±5
^a pVGLUT1lac-Pr&Intron Exp. 2	136±20	95±2	^b ND	^b ND
pVGLUT1lac-BasalPr	117±13	53±4	126±15	48±2
pVGLUT1lac-Pr	48±4	82±1	54±4	19±2
pVGLUT1lac-BasalPr&Intron	55±6	88±2	58±6	17±2

Vectors were injected into POR cortex, and the rats were sacrificed at 4 days after gene transfer. The number of hemispheres analyzed for each vector were: pVGLUT1lac-Pr&Intron Exp. 1, 2 hemispheres; pVGLUT1lac-Pr&Intron Exp. 2, 4 hemispheres; pVGLUT1lac-BasalPr, 4 hemispheres; pVGLUT1lac-Pr, 6 hemispheres; pVGLUT1lac-BasalPr&Intron, 6 hemispheres. The values shown are mean±s.e.m.

^apVGLUT1lac-Pr&Intron Exps. 1 and 2 were previously published (Rasmussen et al., 2007; Zhang and Geller, 2010). pVGLUT1lac-Pr&Intron Exp. 2 was performed in parallel with the other vectors analyzed in this experiment. pVGLUT1lac-Pr&Intron Exp. 1 was performed prior to this experiment, using the same methods.

^bND, not done.

Table 3

Numbers of β -gal expressing cells per hemisphere in rats sacrificed at 4 days after injection of vectors containing specific VGLUT1 promoters/regulatory elements

Vector	β -gal-IR cells / hemisphere	X-gal cells / hemisphere
^a pVGLUT1lac-Pr&Intron Exp. 1		887 $\pm$ 357
^a pVGLUT1lac-Pr&Intron Exp. 2	544 $\pm$ 79	
pVGLUT1lac-BasalPr	486 $\pm$ 56	
pVGLUT1lac-Pr	204 $\pm$ 13	
pVGLUT1lac-BasalPr&Intron	227 $\pm$ 22	

Vectors were injected into POR cortex. For pVGLUT1lac-Pr&Intron Exp. 1, 3 hemispheres were analyzed using X-gal staining, every 4th section was assayed, and the number of X-gal cells was multiplied by 4 to obtain the number of X-gal cells / hemisphere. For the other vector stocks, the numbers of hemispheres analyzed are in the Table 2 notes. For pVGLUT1lac-Pr&Intron Exp. 2, glutamatergic-specific expression was assayed in every 4th section, and the number of β -gal-IR cells was multiplied by 4 to obtain the number of β -gal-IR cells / hemisphere. For the three other vectors, glutamatergic-specific expression was assayed in every 4th section, and GABAergic-specific expression was assayed in every 4th section; the number of β -gal-IR cells in these two assays were added and then multiplied by 2 to obtain the number of β -gal-IR cells / hemisphere. The values shown are mean $\pm$ s.e.m.

^a pVGLUT1lac-Pr&Intron Exps. 1 and 2 were previously published (Rasmussen et al., 2007; Zhang and Geller, 2010). pVGLUT1lac-Pr&Intron Exp. 2 was performed in parallel with the other vectors analyzed in this experiment.