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The use of a viral 2A sequence for the simultaneous over-expression of both the *vgf* gene and enhanced green fluorescent protein (eGFP) *in vitro* and *in vivo*

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HIGHLIGHTS

- The viral 2A sequence is suitable for gene manipulation in the Siberian hamster.
- It allows long-term simultaneous over-expression of 2 genes *in vitro* and *in vivo*.
- We demonstrate dual expression *in vitro* in the neuroblastoma cell line SH-SY5Y.
- We demonstrate dual expression in the hypothalami of Siberian hamsters and mice.

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ABSTRACT

Introduction: The viral 2A sequence has become an attractive alternative to the traditional internal ribosomal entry site (IRES) for simultaneous over-expression of two genes and in combination with recombinant adeno-associated viruses (rAAV) has been used to manipulate gene expression *in vitro*.
New method: To develop a rAAV construct in combination with the viral 2A sequence to allow long-term over-expression of the *vgf* gene and fluorescent marker gene for tracking of the transfected neurones *in vivo*.
Results: Transient transfection of the AAV plasmid containing the *vgf* gene, viral 2A sequence and eGFP into SH-SY5Y cells resulted in eGFP fluorescence comparable to a commercially available reporter construct. This increase in fluorescent cells was accompanied by an increase in VGF mRNA expression. Infusion of the rAAV vector containing the *vgf* gene, viral 2A sequence and eGFP resulted in eGFP fluorescence in the hypothalamus of both mice and Siberian hamsters, 32 weeks post infusion. *In situ* hybridisation confirmed that the location of VGF mRNA expression in the hypothalamus corresponded to the eGFP pattern of fluorescence.
Comparison with old method: The viral 2A sequence is much smaller than the traditional IRES and therefore allowed over-expression of the *vgf* gene with fluorescent tracking without compromising viral capacity.
Conclusion: The use of the viral 2A sequence in the AAV plasmid allowed the simultaneous expression of both genes *in vitro*. When used in combination with rAAV it resulted in long-term over-expression of both genes at equivalent locations in the hypothalamus of both Siberian hamsters and mice, without any adverse effects.

Abbreviations: All standard in this field

1. Introduction

The use of transgenic and knockout mice has contributed greatly to our understanding of gene function and disease. However, these mice models are not only complex to create, but may not be viable, or may produce a complex phenotype reflecting developmental or functional compensation. Gene transfer technologies utilising

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viruses provide a means to overcome some of these limitations. The most widely used viral vectors for neuronal over-expression are recombinant adeno-associated viruses (rAAV), as they have been shown to efficiently and stably transduce a variety of tissues in immunocompetent animals, including mice, rats and Siberian hamsters (Daya and Berns, 2008; Jethwa et al., 2010). However these viruses are limited in their capacity to incorporate foreign DNA by virtue of their genomic size (approximately 5 kb) (Kay et al., 2001). Although hybrids with other viruses have been generated to try to overcome this limitation (Costantini et al., 1999; Nakai et al., 2000; Recchia et al., 1999), the low insert capacity remains an issue if co-expression of genes is required from a single viral vector. For example, the combination of a gene of interest and a reporter gene, such as green fluorescent protein (GFP), is particularly useful in order to enable visualisation of transduced cells both *in vitro* and *in vivo*.

Co-expression of genes can greatly enhance the efficiency and usefulness of transgenic applications. A number of options are available to simultaneously express two genes in particular cells/neurones. Currently the most common approach relies on vector strategies in which genes are linked by an internal ribosomal entry site (IRES) to allow simultaneous expression of genes, since the IRES sequence permits the production of multiple proteins from a single mRNA transcript. Ribosomes bind to the IRES in a 5′-cap independent manner and initiate translation (Jang et al., 1988). However, there are two main limitations to the use of the IRES. First, the size of the IRES is often in excess of 500 base pairs, which further limits the capacity to incorporate foreign DNA. Second, the expression of the downstream gene within the vector is often reduced and therefore expression of the two genes is not equivalent. Recently it has been shown (Chan et al., 2011) that viral 2A sequences can overcome these limitations; with the viral 2A sequence shown to increase reporter gene expression in comparison to the IRES sequence. Thus the viral 2A peptide sequence offers an alternative to the IRES.

The viral 2A sequence was first identified in the picornaviruses (Ryan et al., 1991; Trichas et al., 2008). Viral 2A sequences are relatively short (approximately 20 amino acids) and contain the consensus motif Asp-Val/Ile-Glu-X-Asn-Pro-Gly-Pro (Ibrahimi et al., 2009). The sequence acts co-translationally, the formation of a normal peptide bond between the glycine and proline residues is prevented, which results in ribosomal skipping (Donnelly et al., 2001) and therefore cleavage of the nascent polypeptide. This effect produces multiple genes at equimolar levels. The efficiency of the viral 2A sequence has been demonstrated in a wide range of eukaryotic cells from yeast to mammals (de Felipe and Ryan, 2004), including its effectiveness following insertion between two reporter genes *in vitro* (Ryan and Drew, 1994). Importantly, the viral 2A sequence, in combination with AAV, was able to function in the rat brain without any cytotoxic effects (Furler et al., 2001).

Despite these advantages, the use of viral 2A sequence technology for *in vivo* research has been limited. In this study, we utilised the viral 2A sequence (a) to overcome AAV capacity problems faced utilising large genes and (b) to over-express VGF and eGFP in the hypothalamus of Siberian hamsters and mice to determine its feasibility in standard and non-standard laboratory animals. We clearly demonstrate that the combination of AAV with the viral 2A sequence results in long term over-expression of VGF mRNA and the eGFP reporter gene in the hypothalamus of both species.

Table 1
PCR primers used for the synthesis of VGF-2A fragment, to determine endogenous VGFmRNA and cyclophilin control.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Modified PCR primers (for amplification of VGF cDNA)	CCC GGG AAG CTT **ACC ATG** AAA ACC TTC ACG TTG CCG GCA TCC	GGG CCC TGG GCC AGG ATT CTC CTC GAC GTC ACC GCA TGT TAG CAG ACT TCC TCT GCC CTC TCC ACT GCC GAG CAC GTG CTG CTG CAC CGC CCG
VGF mRNA	GAC CCT CCT CTC CAC CTC TC	ACC GGC TCT TTA TGC TCA GA
Cyclophilin A	TCC TGC TTT CAA GAA TTA TTC C	ATT CGA GTT GTC ACA GTC AGC

The modified PCR primers for the amplification of VGF cDNA (Accession no.: NM_001039385.1) from the pSC-B-AMP/KAN blunt cloning vector to produce the VGF-2A fragment. The forward primer contains a Kozak sequence (in bold) for initiation of translation, while the reverse primer contains the viral 2A sequence (underlined) and a point mutation to allow the removal of the stop codon from the VGF cDNA. Both primers contained unique restriction sites to aid the sub-cloning process. The VGF mRNA and cyclophilin primers were designed from database sequences. Sequence data was input into Primer3 in FASTA format and primers were designed using the default criteria (Primer size; 18–27 bp, Melting temperature 55–62 °C, Amplicon size 50–150 bp) and obtained from Sigma, Dorset, UK.

2. Materials and methods

2.1. Synthesis of construct and viral particles

Construction of the pAAV-CBA-AgRP-IRES-eGFP-WPRE plasmid (from an original plasmid AAV vector, a kind gift from Dr Miguel Sena Esteves, University of Massachusetts, Worcester, USA) has been described previously (Jethwa et al., 2010). We replaced the AgRP-IRES in this plasmid with VGF cDNA and the viral 2A sequence from Trichas et al. (2008) (Fig. 1). Full length mouse VGF cDNA (Accession no.: NM_001039385.1) was isolated and inserted into the blunt cloning vector, pSC-B-AMP/KAN (Agilent Technologies, UK) for amplification using modified PCR primers. The forward primer contained a Kozak sequence for initiation of translation, while the reverse primer contained the viral 2A sequence and a point mutation to allow the removal of the stop codon from the VGF cDNA (Table 1).

The resulting PCR fragment, which contained the VGF cDNA and viral 2A sequence (VGF-2A), was digested with *Hind*III and *Not*I, while the AgRP-IRES sequence was excised from the pAAV-CBA-AgRP-IRES-eGFP-WPRE plasmid vector using *Hind*III and *Age*I, the latter producing an identical 5′ overhang (5′-GGCC-3′) to *Not*I. The digested plasmid was treated with calf intestinal alkaline phosphatase (Promega, USA) to prevent re-ligation of the plasmid without insert. Sure 2 Supercompetent cells (Agilent Technologies, USA) were used for transformation to prevent DNA rearrangement/deletion, as they lack the *Escherichia coli* genes implicated in such events, thereby improving cloning efficiency. The VGF-2A PCR fragment produced by PCR was cloned in to the original AgRP plasmid to produce the pAAV-CBA-VGF-2A-eGFP-WPRE plasmid vector (referred to as pAAV-VGF-GFP) (Fig. 2).

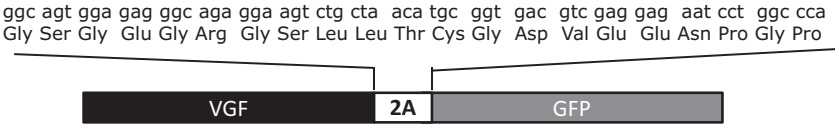

Fig. 1. *The viral 2A sequence*. The viral 2A sequence, containing the consensus motif Asp-Val-Glu-X-Asn-Pro-Gly-Pro, which mediates the co-translational cleavage of the nascent polypeptide, resulting in expression of both the VGF gene and eGFP reporter gene in equimolar amounts. Adapted from Trichas et al. (2008).