Rational design of short constitutive promoters for improved transgene expression in AAV vectors

Greg Nachtrab, Scott Davis, Daniela Martino Roth, Dimitrios Angelis, Alistair Wilson, Claire Geddes, Nandini Narayan, Ranjan Batra, Dan Gibbs

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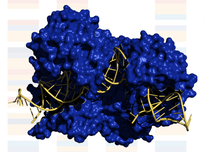

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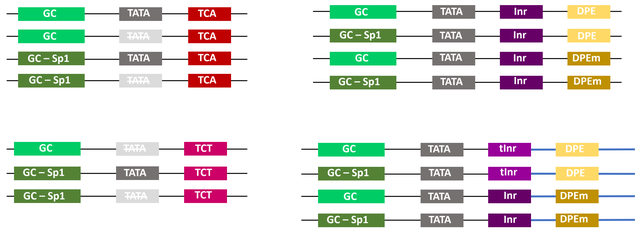
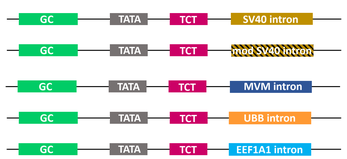
Rationale for engineering short constitutive promoters

- Recombinant AAV vectors are a powerful delivery vehicles for human gene therapy.
- However, the approximately 4.8 kb packaging capacity of AAV vectors significantly limits their use as constructing an expression cassette with a promoter, transgene, and 3'UTR can be challenging.

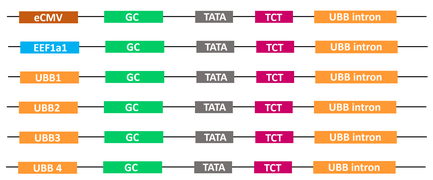
- Current constitutive promoters used in AAV vectors are either long, with lengths greater than 900 bp such as the truncated CAG (tCAG) promoter, or drive weak expression such as the EFS promoter.
- Overexpression of transgenes from the strong CAG promoter has been associated with toxicity *in vivo*
- To expand the utility of AAV vectors we designed a series of short (300-700 bp) constitutive synthetic promoters based on the EFS promoter to generate promoters with a range of expression levels.
- Core promoters are comprised of a number of elements including the GC box, CAAT box, TATA box, Initiator or TCT motif, and the downstream promoter element (DPE).
- The EFS promoter has an identifiable GC box, TATA box, and TCT motif.
- These elements were systematically mutated to either the predicted consensus sequence, enhanced or inactive sequence. Additional novel derivates with DPE sequences were also generated



- Commonly used introns such as the SV40 and MVM introns were inserted in the 5'UTR after the TCT motif. Novel introns derived from the human EEF1A1 and UBB genes were also constructed.



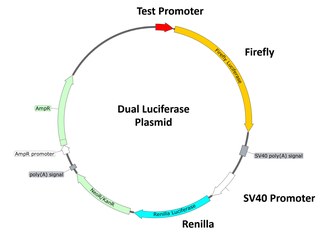
- Based on the results from these promoters, a second series was generated with upstream additions of putative enhancer sequences from UBB, EEF1A1, and CMV.

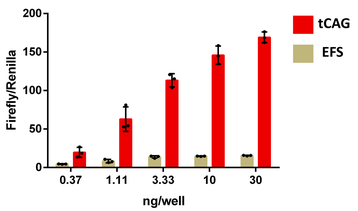


Approach for screening novel promoters

Strength of synthetic promoters assessed with dual luciferase assay

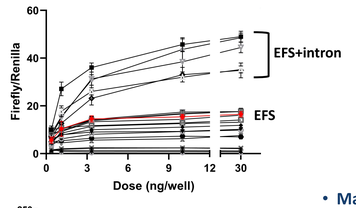
- Promoter variants were cloned into a dual luciferase plasmid. The EFS, tCAG, or novel synthetic promoters drive expression of Firefly luciferase and an SV40 promoter drives Renilla luciferase expression as a transfection control.
- Transfection of HEK293 cells in 96 well plates was performed with five different doses of plasmid.
- 48 hours post transfection the luciferase activity was measured and the ratio of Firefly to Renilla was calculated.
- The assay performed well as there was difference in the strength of the tCAG versus the EFS promoter was readily apparent across doses.

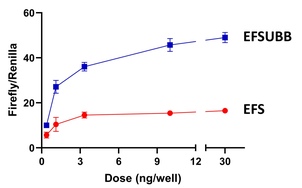


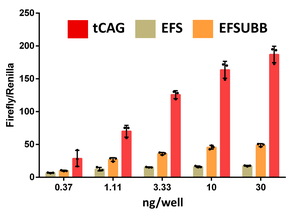


Identification of variants with stronger expression than the EFS promoter

EFS promoters with 5'UTR introns show enhanced expression



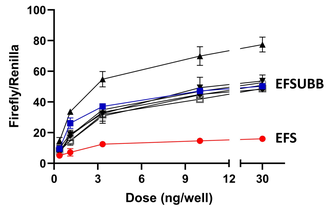


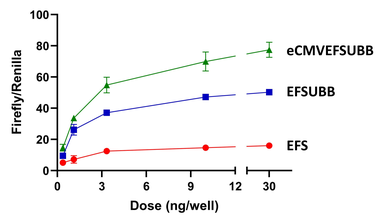


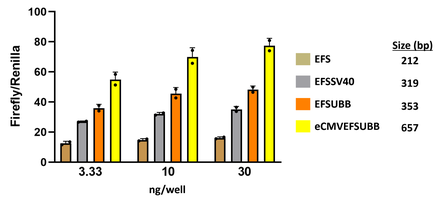
- Majority of EFS based synthetic promoters drive less expression than the original EFS sequence.
- The addition of an intron in the 5'UTR of the EFS promoter significantly boosts expression and improves the dynamic range of expression.
- The UBB derived intron performed best.
- The EFSUBB promoter was approximately 3 times stronger than the EFS promoter.

Generation of a graded series of promoters

Addition of CMV enhancer further increases expression from EFSUBB promoter





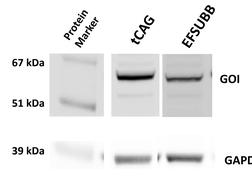


- The addition of an enhancer sequence from CMV further elevates expression from the EFSUBB promoter.
- This creates a series of constitutive promoters with graded levels of expression: tCAG > eCMVEFSUBB > EFSUBB > EFS.

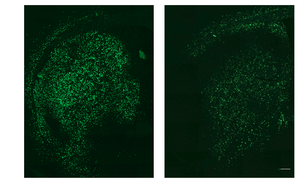
Application of EFSUBB promoter in AAV vectors

EFSUBB drives robust expression from AAV vectors *in vitro* and *in vivo*

AAVRh10 transduction of CHO-Lec2 cells

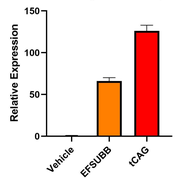


AAVRh10 transduction of mouse striatum



- *In vitro* transduction of CHO-Lec2 cells using AAVRh10 vectors with either the tCAG or EFSUBB promoter shows robust protein expression.
- AAVRh10 EFSUBB drives robust transgene expression *in vivo* following stereotactic injection into the mouse striatum
- EFSUBB driven mRNA expression was approximately 50% of tCAG driven expression at the same vector dose. Transgene overexpression from tCAG promoters can be toxic to neurons *in vivo*
- EFSUBB promoter drives safe and robust transgene expression from an AAV vector in both *in vitro and in vivo* applications.

qPCR of transgene mRNA expression



Summary and Conclusions

- Addition of introns into the 5'UTR region increases both the potency and the dynamic range of the EFS promoter.
- The most effective intron was a novel sequence derived from the human UBB gene.
- The EFSUBB promoter could be further improved by the addition of an enhancer.
- The EFSUBB drives safe and robust expression from AAV vectors in both *in vitro* and *in vivo* in mouse striatum.
- Rationale engineering of promoters can increase the options for expression cassettes in gene therapy applications.