Poster #1277

MEIRAGTx

# Development of rationally designed CAG-based promoters for use in gene therapy

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## ABSTRACT

**Background:** The promoter is an essential cis-regulatory element in any DNA-based gene therapy. It directly controls gene transcription and thereby therapeutic protein expression. In the context of genetic medicines, stronger promoter activity may enable a lower vector dosage of the given gene to achieve therapeutic effect, reducing safety risks associated with high vector dosages, as well as reducing manufacturing costs. To date, an overwhelming majority of promoters used in gene therapy clinical trials are based on cellular CAG or viral CMV promoters. We endeavored to engineer new CAG-based promoters with the aim to increase potency and reduce size to create an improved set of strong, durable constitutive promoters amenable to gene therapy applications. **Methods:** The CAG promoter is a synthetic hybrid promoter consisting of the CMV immediate early enhancer and the promoter, the first exon, and a modified first intron of the chicken beta actin gene. We rationally designed a series of 82 new CAG promoter variants by systematically introducing modifications to each of the promoter elements and tested them in different *in vitro* and *in vivo* models. **Results:** In our library of CAG promoter variants, 51 are smaller than the original CAG, of which 22 are fewer than 1000 base pairs in length. In HEK293T cells, 67 CAG promoter variants were found to be stronger than the original CAG with the strongest promoter exhibiting 13-fold improvement in potency. Two CAG promoter variants, based on improved *in vitro* activity and smaller size (~40% size reduction), were administered by tail vein injection into C57BL/6 mice. Expression in the liver improved by up to 4-fold compared to the original CAG promoter.

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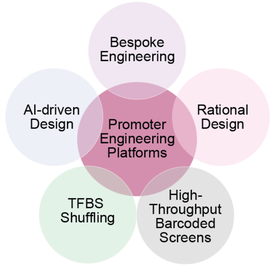
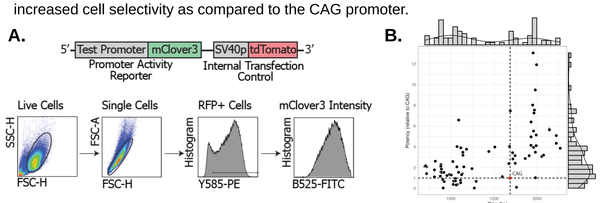
## Promoter engineering can improve strength, specificity, and safety of gene therapies

Benefits of Promoter Engineering for Gene Therapies

- Precise control of therapeutic gene expression[1]
- Cell-specific expression independent of capsid[2]
- Increased potency potentially decreases immune responses and safety risks[3]
- Kinetics impact durability of gene therapy
- Reducing size without sacrificing strength or specificity allows efficient packaging or larger cargo[4]

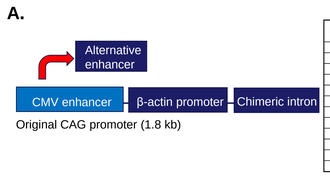



We have built libraries of synthetic promoters and promoter elements available for viral vector optimization. Isolated promoters exhibited a smaller size, improved promoter strength, and/or increased cell selectivity as compared to the CAG promoter.



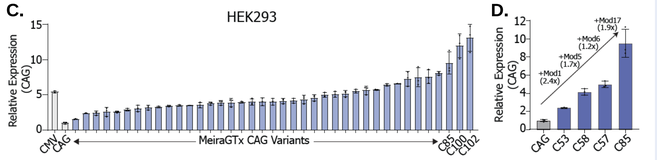

**Development and screening of a diverse CAG promoter variant library. (A)** We used a FACS-based assay to screen promoter activity *in vitro*. Promoter candidates are cloned upstream of mClover3 in a dual-reporter plasmid, which also contains a tdTomato expression cassette that is used as an internal transfection control. Promoter activity is quantified as a ratio of the mean fluorescence intensity of mClover3 and tdTomato in single, live tdTomato+ cells. **(B)** Potency and size distribution of CAG variant library in transfected HEK293 cells.

## Designing smaller or stronger CAG promoter variants



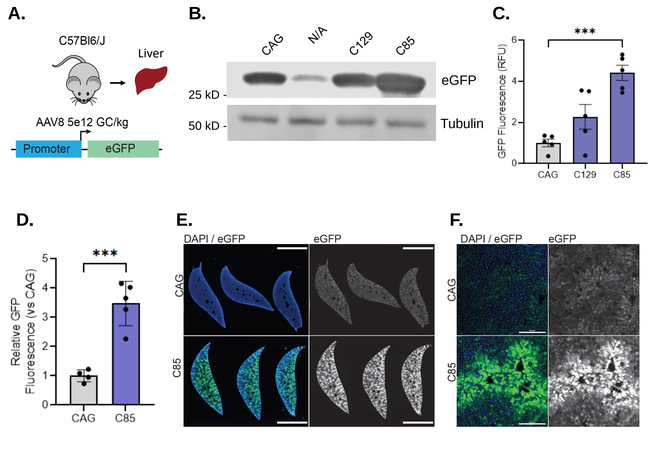

| Name | Promoter | Size (kb) | Neuron BE2-M17 | Neuron N2a | Muscle C2C12 | Kidney HEK293 | RPE ARPE19 | Joint SW982 | Liver HepG2 |
|---|---|---|---|---|---|---|---|---|---|
| C19 | CAG | 1.8 | 0.9 | 0.9 | 0.8 | 0.4 | 0.4 | 0.3 | 0.5 |
| C20 | CBh | 0.8 | 1 | 1 | 1 | 1 | 1 | 1 | 1.0 |
| C21 | ENHANCER1-CBh | 0.9 | 2.8 | 2.5 | 1.4 | 0.6 | 0.7 | 1.1 | 1.3 |
| C22 | ENHANCER2-CBh | 0.9 | 1.7 | 1.7 | 0.8 | 0.8 | 0.6 | 0.5 | 0.8 |
| C23 | ENHANCER3-CBh | 0.9 | 2.5 | 2.4 | 1 | 0.6 | 0.7 | 0.8 | 0.6 |
| C24 | ENHANCER4-CBh | 0.9 | 1.7 | 2.1 | 0.9 | 0.9 | 0.6 | 0.4 | 0.5 |
| C25 | ENHANCER5-CBh | 0.9 | 2.4 | 1.5 | 0.9 | 0.6 | 0.6 | 0.6 | 0.6 |
| C26 | ENHANCER6-CBh | 0.9 | 3.6 | 2.5 | 0.8 | 0.8 | 0.7 | 0.9 | 0.8 |
| C27 | ENHANCER7-CBh | 0.9 | 2 | 2.9 | 1 | 0.7 | 0.6 | 0.3 | 1.0 |
| C28 | ENHANCER8-CBh | 0.7 | 1.5 | 1.9 | 0.9 | 0.8 | 0.5 | 0.6 | 0.8 |
| C29 | ENHANCER9-CBh | 0.7 | 1.1 | 1.8 | 0.8 | 0.8 | 0.4 | 0.6 | 0.8 |
| C30 | ENHANCER10-CBh | 0.7 | 1.1 | 1.4 | 0.9 | 0.8 | 0.3 | 0.4 | 0.7 |

| Promoter | CMV | CAG | C53 | C54 | C55 | C56 | C57 | C58 | C63 | C64 | C65 | C66 | C67 | C68 | C69 | C70 | C83 | C84 | C85 | C86 | C87 | C88 | C89 | C90 | C91 | C99 | C100 | C101 | C102 | C108 | C110 | C173 | C174 | C175 | C176 | C177 | C178 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Size (kb) | 0.7 | 1.8 | 1.7 | 2.1 | 1.9 | 1.7 | 1.9 | 1.9 | 2.0 | 1.7 | 1.5 | 1.1 | 1.7 | 1.4 | 1.2 | 1.1 | 1.9 | 2.0 | 2.0 | 2.0 | 2.0 | 1.9 | 2.0 | 2.0 | 2.2 | 2.2 | 2.0 | 2.0 | 2.0 | 1.7 | 1.1 | 1.1 | 1.0 | 1.1 | 1.1 | 1.1 | 1.1 |
| Potency (HEK293T) | 5.3 | 1.0 | 2.4 | 3.1 | 2.9 | 2.4 | 5.0 | 4.2 | 5.5 | 3.4 | 4.0 | 3.2 | 4.0 | 3.6 | 3.8 | 4.0 | 6.4 | 4.1 | 9.5 | 5.2 | 4.0 | 8.0 | 3.5 | 7.6 | 5.1 | 4.4 | 11.9 | 7.3 | 13.1 | 7.5 | 0.8 | 1.5 | 2.3 | 6.6 | 3.7 | 3.5 | 2.0 |
| Potency (Huh7) | 8.1 | 1.0 | 1.7 | 4.1 | 2.9 | 1.8 | 9.5 | 9.2 | 8.1 | 6.5 | 5.4 | 5.2 | 7.4 | 7.6 | 7.4 | 7.1 | 9.8 | 4.2 | 10.9 | 3.5 | 2.4 | 10.2 | 2.3 | 9.2 | 3.6 | 4.2 | 8.8 | 7.5 | 9.7 | 2.9 | 1.4 | 2.6 | 2.2 | 6.0 | 1.9 | 1.2 | 2.5 |



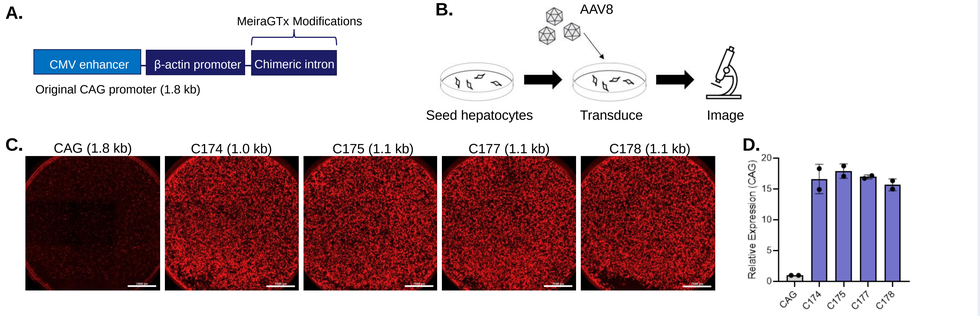

**MGTx promoter engineering generates CAG variants that are up to 13-fold more potent than CAG. (A)** Approach 1: CMV enhancer was replaced to create novel CAG variants. Heatmap shows size (blue) and activity (green) of CAG variants in various cell lines using our dual-reporter assay. **(B)** Approach 2: CAG variants were designed by modifying the promoter and/or intron regions. Heatmap shows size (blue) and activity (green) of CAG variants in HEK293 and Huh7 cells *in vitro*. **(C)** Screening in transiently transfected HEK293 cells identifies candidates that are up to 13-fold stronger than the original CAG promoter. **(D)** Example of the step-wise evolution of one top variant that is ~9-10 fold stronger than the original CAG.

## MGTx-C85, a CAG variant, is highly potent in the mouse liver




**MGTx-C85 promoter is 4-fold stronger than CAG in the mouse liver. (A)** Promoter activity was tested in the mouse liver by injecting AAV8-eGFP (5e12 GC/kg) in male mice, aged 8 weeks (n=5 per group). The liver was collected 4 weeks later. **(B)** Western blot of liver lysate. **(C)** Native eGFP fluorescence was quantified from liver lysate. **(D)** Quantification and **(E)** representative images of native eGFP fluorescence in serial liver sections. Left image is merged DAPI (blue) and eGFP (green). Right image is eGFP signal alone (grayscale). Scale bar: 5 mm. **(F)** Representative images of transduced liver tissue at 20x. Scale bar: 200 µm.

## Smaller CAG variants are >15x stronger than CAG in transduced hepatocytes




**MGTx promoter engineering generates CAG variants that are over 15-fold more potent than CAG in hepatocytes. (A)** The intron region of CAG was modified to create MGTx promoters C174-C178. **(B)** Primary mouse hepatocytes were transduced with AAV8-miRFP713-WPRE-SV40polyA at 10,000 multiplicity of infection. After 7 days, cells were imaged and miRFP713 fluorescence was quantified. **(C)** Representative images of transduced hepatocytes. Scale bar: 200 µm. **(D)** MGTx promoters have over 15-fold higher relative fluorescence compared to the original CAG in primary mouse hepatocytes.

## Conclusions

- We designed a library of synthetic CAG promoter variants that are up to 15-fold stronger than the original CAG promoter .
- Modifying the CMV enhancer generates CAG variants that are smaller but maintain promoter activity. Some variants show increased activity in select cell types.
- Step-wise evolution of MGTx-C85 leads to a highly potent promoter that is ~4-fold stronger than CAG in the mouse liver.
- Four MGTx variants are ~800 bp smaller than CAG but exhibit 15-fold higher expression in primary mouse hepatocytes.
- Successfully rationally designed a diverse set of CAG promoter variants that are stronger and/or smaller.

## References

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4. Wu et al. Effect of genome size on AAV vector packaging. *Mol Ther*. 2010 Jan; 18(1): 80–86.