Poster # 1466

MEIRAGTx

Identification of novel inflammation-inducible promoters using a hybrid-barcoded SuRE™ library

Kassiani Kytidou[1], Sabine van der Sanden[1], Bart Kok[1], Marleen van Loenen[1], Alexandria Forbes[1], Joris van Arensbergen[2] & Janneke Meulenberg[1]

[1] MeiraGTx, Paalbergweg 2-4, 1105 AG, Amsterdam, The Netherlands

[2] Annogen, Amsterdam Science Park 406, 1098XH, Amsterdam, The Netherlands

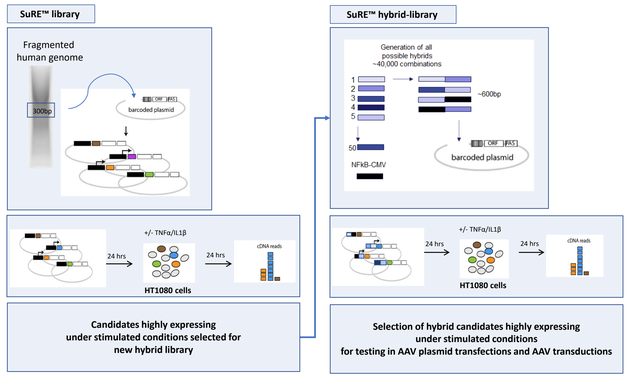
ABSTRACT

AAV-based gene therapy vectors are promising candidates for the treatment of inflammatory disease that result from a biological response of the immune system triggered by a variety of different factors. Regulatory elements, including promoters and enhancers, are engineered for use in AAV vectors to optimize the strength, kinetics, and specificity of transgene expression. The incorporation of promoters inducible by inflammation will help to reduce the risk for side effects due to overexpression of and/or continuous exposure to the anti-inflammatory therapeutic protein by such AAV vectors. The aim of this study was to identify inflammation-inducible hybrid-promoters and/or cis-regulatory elements that show improved inducibility and/or higher expression compared to the reference inflammation-inducible promoter NFkB-responsive CMV (NFkB-CMV promoter). To this end, Annogen's Survey of Regulatory Elements (SuRE™) methodology was applied to identify new cis-regulatory elements in the human genome. A barcoded library containing around 300 million human DNA fragments/elements, with an average insert size of about 300 base pairs (bp), was generated and used to transfect HT1080 cells. Transfected HT1080 cells with and without stimulation with TNF alpha (TNFα) and IL-1 beta (IL-1β) human recombinant cytokines were analyzed for expression levels of the ~300 million elements as compared to their frequency in the input library to generate a genome-wide profile. Elements displaying high expression in stimulated conditions compared to unstimulated conditions were selected from the SuRE™ screening and combined with the reference NFkB-CMV promoter to generate a new barcoded library, consisting of ~40,000 new hybrid combinations, each of around 600 bp in size. The new barcoded hybrid-library was subsequently used in a second round of screening in HT1080 cells. The best performing hybrid-elements were selected for further analysis in the context of the AAV2 genome upon plasmid transfection and AAV transduction, with luciferase as reporter protein. Primary cells and different cell lines were used to determine the strength and inducible character of the new hybrid promoters. The expression profiles from plasmids and AAV viruses revealed a number of new hybrid promoter elements that displayed improved inducibility and/or higher expression under inflammatory conditions compared to the reference NFkB-CMV promoter.

1. SuRE™ screenings

First SuRE™ screening in HT1080 under cytokine stimulation.

Second SuRE™ screening in HT1080, using a hybrid-barcoded plasmid library.

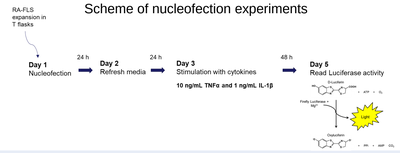


- Annogen's SuRE™ methodology was applied to screen for cis regulatory elements.
- Human genomic DNA was randomly fragmented. Fragments of around 300 bp were ligated into barcoded plasmids, generating a barcoded plasmid library.
- A barcode to fragment association table was generated after sequencing of plasmid library.
- HT1080 cells were transfected with the barcoded library in the presence or absence of inflammatory cytokines (TNFα and IL-1β).
- cDNA of transfected cells was isolated under stimulated and non stimulated conditions, following sequencing of barcoded area. Normalization of expression with plasmid DNA 'input' followed.
- Elements were selected showing high expression under stimulated conditions.
- The selected elements were used, together with the NFkB-CMV promoter, in the generation of a new hybrid like barcoded SuRE™ library. Around 40,000 hybrid combinations were made and ligated into the barcoded plasmids.
- A new round of plasmid transfections was conducted, similar to the first screening and candidates were selected for further evaluation in AAV plasmid transfections and AAV transductions.

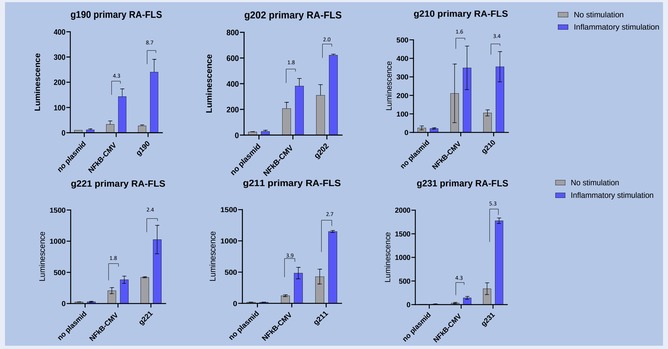
2. Plasmid transfections of primary RA-FLS cells

Composition of AAV plasmid cassette

Scheme of nucleofection experiments



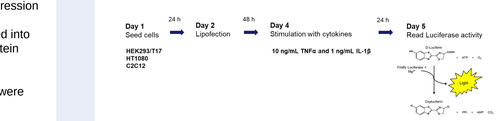
- Hybrid promoter candidates were selected from second SuRE™ screening, based on high expression during stimulated conditions.
- Hybrid candidates were cloned into expression cassettes and protein expression was evaluated.
- Primary Rheumatoid arthritis fibroblast like (RA-FLS) cells were nucleofected with selected candidates.
- Data are presented for select hybrid promoters that showed particularly high increases in expression in response to inflammatory stimuli.



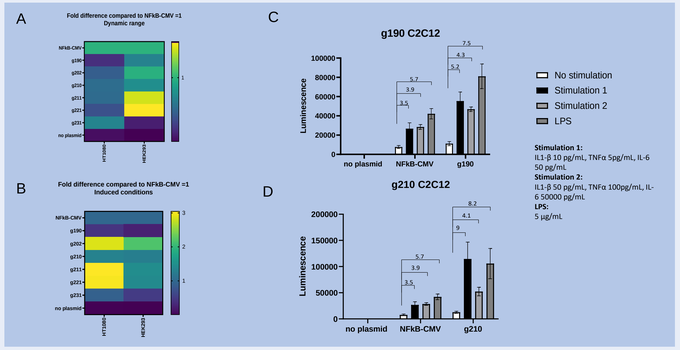
Primary RA-FLS cells were nucleofected with plasmids containing the hybrids promoters, and Luciferase expression was measured. The 6 hybrid promoters are showing 2- to 11-fold higher absolute expression during stimulated condition as well as 2- to 3-fold improved dynamic range (i.e., difference in expression between unstimulated and stimulated condition) compared to NFkB-CMV promoter.

3. Plasmid transfections of cell lines

Scheme of lipofection experiments



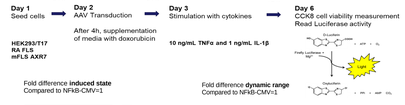
- Selected hybrid candidates were also screened using HEK293T/17 and HT1080 cells. Cell lines were transfected using lipofectamine reagent (A and B).
- Data are presented for select hybrid promoters that showed particularly high increases in expression in response to inflammatory stimuli (A and B).
- In addition, candidates were also screened using different stimulation conditions in order to mimic *in vivo* cytokine levels, using the murine muscle cell line C2C12. Data of g190 and g210 are shown (C and D).

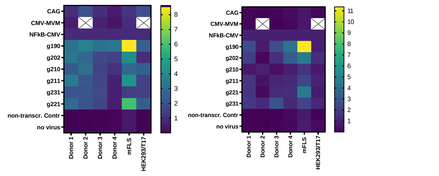


Lipofections of HT1080, HEK293T/17 and C2C12 cells. (A) Fold difference of absolute expression, and (B) fold difference of dynamic range of hybrid promoters under inflammatory versus non-inflammatory conditions compared to NFkB-CMV promoter (expression set at 1). Candidates show improved absolute expression under stimulation and / or improved dynamic range. (C) Stimulation of hybrid promoters with different cytokine concentrations in order to mimic cytokine *in vivo* levels. LPS was used as a positive control. g190 and g210 show high induction levels under low physiological cytokine doses.

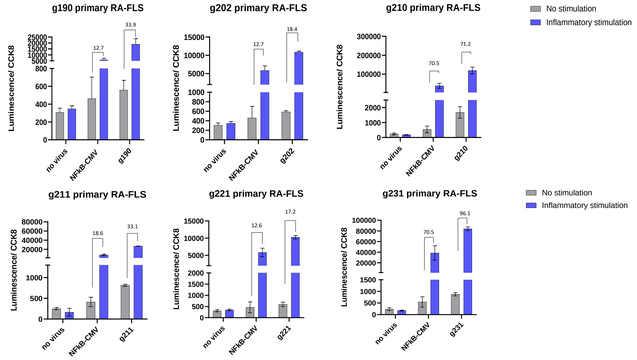
4. AAV transductions of primary RA-FLS cells

Scheme of AAV transductions





- AAV6 viruses were produced harboring the different hybrid promoters.
- Viruses were used to transduce primary RA-FLS cells (4 different donors), mouse FLS (mFLS) and HEK293T/17 cells and Luciferase expression was measured.
- Data are presented for select hybrid promoters that showed particularly high increases in expression in response to inflammatory stimuli.
- The hybrid promoters show higher absolute expression under stimulation, as well as dynamic range compared to NFkB-CMV.



5. Summary

- SuRE™ screenings performed in HT1080 cells in the presence or absence of inflammatory cytokines (TNFα and IL-1β), led to the successful identification of novel potent inflammation inducible promoters.
- A subset of these promoters was tested for transgene expression using AAV based plasmid transfections and AAV transductions.
- They displayed higher expression during stimulated state and/or improved dynamic range compared to the NFkB-CMV benchmark promoter in both transfections and transductions of primary RA-FLS.
- They also showed high responsiveness to cytokine levels that mimic *in vivo* levels in the muscle cell line C2C12.