Rational Design and Cloning of a Stable RPGR ORF15 cDNA Encoding the Full-length Native RPGR protein

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BACKGROUND

X-linked retinitis pigmentosa (XLRP) is an inherited condition that causes progressive vision loss in males. The condition begins with night blindness in young boys followed by progressive constriction of the field of vision. Affected men become legally blind at an average of about 45 years of age.

The most common form of XLRP is caused by mutations in the retinitis pigmentosa GTPase regulator (RPGR) gene, and AGTC is developing a gene therapy product for this form of XLRP.

Alternative splicing of the RPGR gene results in expression of multiple isoforms of the RPGR protein. Only the isoform C is predominantly expressed in photoreceptors.

The RPGR cDNA contains a long purine-rich repetitive sequence in the ORF15 exon that is unstable during recombinant DNA manipulation . This complicates efforts to develop AAV-based vectors for gene therapy of XLRP caused by RPGR mutations. We reasoned that the stability of RPGR-ORF15 cDNA could be improved significantly by rational design of the cDNA sequence through codon modification without changing the amino acid sequence.

METHODS

Design of the stable RPGR gene is based on NCBI reference sequence NM_001034853, the transcript variant C mRNA of RPGR (**Figure 1**).

Genetic codon usage of the coding gene was optimized for human cell expression. After codon optimization:

- The Codon Adaptation Index (CAI) increased from 0.73 to 0.87
- The Frequency of Optimized Codons (FOP) increased from 32% to 57%
- The GC content was adjusted from 47.3% to 56.1% and was more evenly distributed
- Potential CIS-acting elements, e.g. polyA (AATAAA, AAAAAAA) and polyT (TTTTTT) were removed
- Some conflicting restriction enzyme sites were removed
- The maximum repeat size decreased from 50bp to 17bp

Repetitive sequences, especially in the purine-rich region, were further modified to minimize frequency of repeat sequences. The redesigned RPGR (RPGRco) encodes a full-length human RPGR isoform C protein and the 1152 amino acid sequence is identical to the NCBI reference sequence NP_001030025 and CCDS 35229.1. The RPGRco cDNA was synthesized and first cloned in a conventional cloning vector and then into an AAV vector plasmid used for AAV production. The RPGRco expression cassette was further cloned into a recombinant herpes simplex (HSV) vector for large scale AAV production. AAV vectors containing the RPGRco expression cassette were produced by both a plasmid transfection method and the AGTC proprietary herpes simplex virus (HSV) based manufacturing system. Stability of RPGRco cDNA was verified by DNA sequencing of plasmid preparations at each step of cloning and AAV vector preparations produced by both a plasmid transfection method or the HSV-based AAV manufacturing system.

RESULTS

- The RPGR-OFR15 coding sequence was redesigned and synthesized (RPGRco).
- The redesigned gene was stable during multiple rounds of subcloning in plasmids:
  - Subclones of a plasmid containing RPGRco showed uniformed restriction enzyme digestion maps (**Figure 2**).
  - The plasmid carrying RPGRco was readily produced with good yield in bacteria grown at 37°C (data not shown). Unstable DNA sequences normally require special conditions, such as lower temperature, lower density, etc.

RESULTS (continued)

- The RPGRco expression cassette was cloned into a large recombinant HSV vector and expanded through multiple passages without signs of instability (data not shown).
- AAV vectors were successfully produced from HEK293 cells transfected with a plasmid carrying the RPGRco cDNA and the yield was comparable to other stable genes of interest (data not shown).
- Large scale production of AAV was successful using the AGTC proprietary infection method with a recombinant HSV carrying the RPGRco cDNA and the yield was comparable to other stable genes of interest (data not shown).
- DNA sequences of the entire RPGRco gene were 100% identical on multiple clones of plasmids, recombinant HSV and AAV products (**Figure 3**).

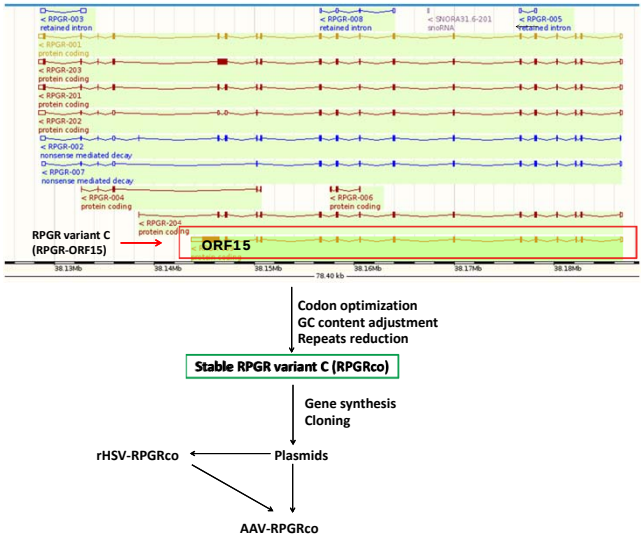

Figure 1. Rational design of a stable RPGR-ORF15 cDNA. The design is based on the published sequence of RPGR variant C, which differs from other variants by including a large ORF15 exon. The purine-rich repetitive sequence in ORF15 is the cause of an unstable RPGR gene and is also the mutation hot spot that results in XLRP. Our design strategy reduced the size of repeats and optimized codon usage for human expression. The redesigned RPGRco was then synthesized, cloned into various vectors including plasmid, HSV and AAV. Stability of the gene was verified by DNA sequencing of these products.

RESULTS (Continued)

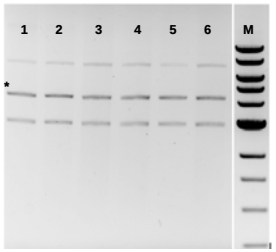

Figure 2. Subcloning of pTR-IRBP-RPRGco. The plasmid was transformed into SURE2 competent cells and subclones 1-6 were selected and grown at 37°C overnight. The plasmid DNAs were extracted and analyzed in agarose gel electrophoresis after Sma I cleavage. The asterisk * indicates the band that contains redesigned RPGRco, which is correct in size (4.2kb) and uniform for all subclones. Normally, when a particular DNA sequence is unstable, the corresponding band would change in size or become a smear in subclones. The lane on the right (M) is the 1kb DNA molecular size ladder from New England Biolabs.

Figure 3 DNA sequence alignment of a stretch of the repetitive purine-rich region of plasmids and AAV vectors containing the RPGR gene. The redesigned hRPGRco cDNA sequence for gene synthesis (bottom), and the DNA sequencing data obtained from a cloned pTR-RPGRco plasmid (top), 3 lots of AAV made by a plasmid transfection method and 1 lot of AAV2tYF-RPGRco made by the HSV-based AAV manufacturing system were all identical.

CONCLUSIONS

- A stable RPGR cDNA that encodes full-length, native RPGR protein was obtained and will allow large scale production of an AAV vector for treatment of patients with XLRP.

REFERENCES

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2. Deng WT et al. Stability and safety of an AAV vector for treating RPGR-ORF15 X-linked retinitis pigmentosa. Hum Gen Ther 2015; 26:593-602

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