Design of non-viral vector with improved regulatory features towards therapeutic application

B. Sharan Sharma¹², Vaishna Prabhakaran¹, Ramtej J. Verma²,*

¹Indrashil Institute of Science & Technology (IIST)/Indrashil University (IU), Kadi, Mehsana – 382740, Gujarat, India; ²Department of Zoology, Biomedical Technology & Human Genetics, University School of Sciences, Gujarat University, Navrangpura, Ahmedabad – 380009, Gujarat, India; Ramtej J. Verma - E-mail: rjvermasosgu@gmail.com; Tel: +91-79-26302362; Fax: +91-79-26303196

ORCiD(s): B. Sharan Sharma: https://orcid.org/0000-0002-4317-468X; Ramtej J. Verma: https://orcid.org/0000-0001-9219-8074

Received February 3, 2020; Accepted April 2, 2020; Published April 30, 2020

DOI: 10.6026/97320630016307

Declaration on Publication Ethics:
The authors state that they adhere with COPE guidelines on publishing ethics as described elsewhere at https://publicationethics.org/.
The authors also undertake that they are not associated with any other third party (governmental or non-governmental agencies) linking with any form of unethical issues connecting to this publication. The authors also declare that they are not withholding any information that is misleading to the publisher in regard to this article.

Declaration on official E-mail:
The corresponding author declares that official e-mail from their institution is not available for all authors

Abstract:
Viral vectors based gene therapy is often compromised by adverse immunological reactions raising safety concerns. Hence, improved design and development of non-viral vectors with strong regulatory regions is desired. We describe the design of a non-viral mammalian expression vector in which the primary transgene (a truncated dystrophin gene linked with Duchenne muscular dystrophy (DMD)) named microdystrophin delR4-R23/delCT (MD1) is under the transcriptional control of elements of desmin locus control region (DES-LCR). The designed vector, named as DES-LCR/MD1-EGFP, was constructed by cloning two fragments into the pBluescript backbone. Fragment 1 contains DES-LCR enhancer and DES-LCR promoter region while fragment 2 contains MD1 transgene and reporter EGFP (enhanced green fluorescent protein) gene separated by linker P2A (2A peptide). This vector design provides a framework for strong regulation with non-viral features. This design forms the foundation for application in conditions linked to multisystem diseases.

Keywords: DES-LCR, Microdystrophin, DMD, Non-viral vector, Gene therapy

Background:
Applications of gene therapy are tremendous and offers hope to treat genetic diseases at large. Success of gene therapy lies on the design, development and delivery of vectors, which can be of viral and non-viral types. Both, viral and non-viral gene therapy vectors, have applications in preclinical and clinical settings. Viral vectors have emerged as effective gene therapy vehicles for clinical gene therapy [1], however, safety has been an issue on use of viral vectors since they may generate strong immune response [2]. On the other hand, non-viral vectors have been ignored in the past but they certainly represent the long-term future of gene therapy owing to their increased safety. In recent years, interest towards
development of non-viral DNA vectors has progressed steadily [3], and several non-viral vector systems have been developed and successfully employed for safe delivery [4]. There are opportunities for further development of non-viral vectors with improved design to address therapeutic needs. One of the solutions to improve the design includes inclusion of strong regulatory features to achieve desired expression of transgenes.

Role of cis-regulatory elements, as strong regulatory sequences, in the design of gene therapy vectors has long been known [5-6]. Sequences of locus control regions (LCRs), non-coding cis-regulatory regions, have been used in the past in viral vectors to control expression of transgenes [7-8]. Ability to control gene expression at ectopic sites makes LCRs unique and different from other distal elements of the genome. Unique regulatory signatures have been identified in the human LCR sequences [9], and further efforts to use these special regulatory elements in the design of gene therapy vectors will pave the way to generate new generation of gene therapy vectors with increased safety.

It is of interest to design a non-viral mammalian expression vector for the expression of a truncated version of dystrophin gene, microdystrophin delR4-R23/delCT (MD1). Typically, truncated versions of dystrophin gene are preferred choice for gene therapy of DMD [10]. Despite the promise of gene therapy for the treatment of DMD, it has been challenging to achieve optimum and prevalent expression of therapeutic gene [11]. Hence, we describe the design of a non-viral vector with improved regulatory features using computer aided sequence analysis tools.

**Methodology:**

**Vector design:**
SnapGene v5.0 software (from GSL Biotech; available at snapgene.com) was used to rationally design a non-viral mammalian expression vector containing elements of human LCR (DES-LCR) for expression of transgenes (MD1 and EGFP). pBluescript plasmid (3931bp) (adapted from Addgene plasmid #24595) was used as empty backbone to construct the vector. Features of this empty backbone are listed in Table 1.

**Restriction and insertion cloning of fragments into empty backbone:**
Two fragments were cloned into the empty backbone of pBluescript at different sites using restriction and insertion cloning feature of SnapGene. Fragment 1 (1069bp), containing sequences of DES-LCR enhancer region and DES-LCR promoter region, was cloned at the ApaI (21) and Hind III (257) restriction sites. DES-LCR enhancer (758bp) and promoter (311bp) sequences were retrieved from NCBI (accession numbers NG_046330.1 (17788-18539) and NG_046330.1 (18540-18844) respectively). ApaI and Hind III restriction sequences were added into the 5’ and 3’ ends respectively of fragment 1 before cloning.

Fragment 2 (4396bp), containing sequences of microdystrophin delR4-R23/delCT (MD1) gene obtained from Foster et al. [12], linker P2A (2A peptide), and reporter EGFP (enhanced green fluorescent protein) gene, was cloned at the EcoRV (265) and BsaBI (826) restriction sites. MD1 is a truncated version (3612bp) of the dystrophin gene used for gene therapy of Duchenne muscular dystrophy (DMD). MD1 sequence was retrieved from Foster et al. [12] which is a codon optimized sequence for maximal expression. P2A (57bp) and EGFP (727bp) sequences were taken from Addgene plasmid #111814. EcoRV (265) and BsaBI (826) restriction sequences were added into the 5’ and 3’ ends respectively of fragment 1 before cloning. Constructed vector (with cloned fragments) was named as DES-LCR/MD1-EGFP.
Amplification of MD1 gene:
From the constructed DES-LCR/MD1-EGFP vector sequence, forward and reverse primers, MD1 F (24bp) and MD1 R (29bp) respectively, were designed using the ‘add primer’ option of SnapGene for the in-silico amplification of MD1 gene. Restriction sites were added in the primers for future cloning of the MD1 gene. MD1 F contain EcoRV restriction site and MD1 R contain HindIII restriction site (Table 2). 'PCR' feature of SnapGene was used to amplify the MD1 gene from the constructed DES-LCR/MD1-EGFP vector. MD1 F and MD1 R primers were selected and in-silico PCR was run. Further, ‘simulate agarose gel’ option was used to confirm the size of amplified MD1 gene on 1% agarose.

Translation of transgenes:
MD1 and EGFP transgenes were translated to generate amino acid products using the ‘translation feature’ of SnapGene and HindIII are also present at the start and end, respectively, of the amplified sequence for future cloning purposes.

Results:

Designed DES-LCR/MD1-EGFP vector:
Non-viral mammalian expression vector, constructed by cloning two different fragments into the pBluescript backbone using SnapGene, was named as ‘DES-LCR/MD1-EGFP’ vector. DES-LCR/MD1-EGFP is an 8588bp long circular vector characteristics of which are given in Table 3. DES-LCR/MD1-EGFP is a high copy number vector for growth in bacteria and contains Ampicillin resistance gene, which confers resistance to antibiotic Ampicillin for selection during bacterial growth. Polyadenylation signal from SV40 poly(A) sequence of the vector helps in addition of poly(A) tail to generated mRNAs from the transgenes during propagation in mammalian cells.

Amplified MD1 gene:
MD1 gene was in-silico amplified using designed MD1 F and MD1 R primer pair. MD1 F (24-mer) and MD1 R (29-mer) primers bind to the 1086 to 1109 and 4675 to 4697 binding sites respectively in the DES-LCR/MD1-EGFP vector with a Tm of 64°C and 60°C respectively. Both these primers contain specific restriction sites for future cloning purposes. Upon in-silico PCR, MD1 F and MD1 R primers generated expected 3.6kb amplicon/product size of which was confirmed by checking the position of ampiclon on ‘simulated

Table 1: Major elements of the pBluescript empty backbone (3931bp)

| Element                        | Location       | Size (bp) |
|--------------------------------|----------------|-----------|
| SV40 poly(A) signal           | 928 to 1062    | 135       |
| T3 promoter                   | 1108 to 1126   | 19        |
| Lac operator                  | 1171 to 1187   | 17        |
| Lac promoter                  | 1195 to 1225   | 31        |
| CAP (catabolite activator protein) binding site | 1240 to 1261 | 22 |
| Ori (Origin of replication)   | 1349 to 2137   | 589       |
| AmpR (Ampicillin resistance)  | 2308 to 3168   | 861       |
| AmpR promoter                 | 3169 to 3273   | 105       |
| F1 ori (F1 bacteriophage origin of replication) | 3299 to 3731 | 453 |

Table 2: Cloned fragments in the DES-LCR/MD1-EGFP vector:
Fragment 1 in the vector is located form position 23 to 1079 and fragment 2 is located from position 1092 to 5478. Components of fragment 1, DES-LCR enhancer and promoter, are located from 23 to 774 and 775 to 1079 respectively (Table 4). Components of fragment 2, MD1 gene, P2A sequence and EGFP gene, are located from 1092 to 4697, 4699 to 4755 and 4762 to 5478, respectively. Map of the vector is given as Figure 1.

Table 2: Designed primers for MD1 gene amplification

| Primer | Sequence (5’ to 3’) | Length | Added Restriction Site |
|--------|---------------------|--------|------------------------|
| MD1 F  | GATATCCACACATCGGCGG | 24-mer | EcoRV                  |
| MD1 R  | AAGCTTAACATCACATGCG | 29-mer | HindIII                |

Table 3: Major elements of the pBluescript backbone (3931bp)

| Element                        | Location       | Size (bp) |
|--------------------------------|----------------|-----------|
| SV40 poly(A) signal           | 928 to 1062    | 135       |
| T3 promoter                   | 1108 to 1126   | 19        |
| Lac operator                  | 1171 to 1187   | 17        |
| Lac promoter                  | 1195 to 1225   | 31        |
| CAP (catabolite activator protein) binding site | 1240 to 1261 | 22 |
| Ori (Origin of replication)   | 1349 to 2137   | 589       |
| AmpR (Ampicillin resistance)  | 2308 to 3168   | 861       |
| AmpR promoter                 | 3169 to 3273   | 105       |
| F1 ori (F1 bacteriophage origin of replication) | 3299 to 3731 | 453 |

Table 4: Designed primers for MD1 gene amplification

| Primer | Sequence (5’ to 3’) | Length | Added Restriction Site |
|--------|---------------------|--------|------------------------|
| MD1 F  | GATATCCACACATCGGCGG | 24-mer | EcoRV                  |
| MD1 R  | AAGCTTAACATCACATGCG | 29-mer | HindIII                |
agarose gel image. Map of amplified MD1 gene highlighting presence of various restriction sites is given as Figure 2.

| Fragment | Size (bp) | Components | Location |
|----------|----------|------------|----------|
| **Fragment 1** | 1069bp | DES-LCR Enhancer, Microdystrophin delR4-R23/delCT | 23 to 774 |
| | | P2A (2A peptide) | 775 to 1079 |
| | | EGFP (Enhanced Green Fluorescent Protein) | 3612 to 4697 |
| **Fragment 2** | 4396bp | P2A | 1069 to 4697 |
| | | EGFP (Enhanced Green Fluorescent Protein) | 4699 to 4755 |
| | | **Total** | 4762 to 5478 |

Translated transgenes:
Upon translation, open reading frames (ORFs) of the transgenes MD1 (3594bp) and EGFP (717bp) generated 1197aa (137.8kDa) and 239aa (26.9kDa) long products, respectively.

**Discussion:**
With viral vectors dominating cell and gene therapy, non-viral vectors sidestep the main concerns that come with using viruses: safety, immunogenicity and manufacturing limits (yield, scaling-up and costs) [13]. New generation of vectors with increased safety are desirable for gene therapy of constitutional disorders to achieve permanent genetic modification and stable expression of transgenes. Introduction of novel and strong regulatory elements in the non-viral vectors provides a solution towards improved design of gene therapy vectors.

LCRs are unique non-coding regulatory sequences with their ability to control gene expression at ectopic locations. These regulatory sequences have not been studied much in the past, however, their presence in the mammalian genomes makes them important. LCRs have the ability to enhance the expression of linked genes to physiological levels indicating that they play a significant role in controlling the expression of target genes [14]. Potential of using LCR elements in vector design for expression of transgenes have been realized in the past [7-8, 15]. However, majority of them are viral vectors. In this work, we designed a non-viral mammalian expression vector via computer-aided tools expressing transgenes under control of elements of LCR.

Despite the availability of gene therapy for the treatment of DMD, many current challenges associated with the therapy are yet to be overcome. Hence, improved next-generation vectors to overcome some challenges of gene therapy for gene diseases like muscle disorders are urgently needed. Computational tools have proven to be very powerful in the systematic and rational design and analysis of vectors of users' choice [16,17]. Recently, Sarcar et al. [18] reported muscle-directed gene therapy by in silico vector design. They used AAV vector in combination with novel cis-regulatory modules (CRMs), containing clusters of TFBSs, to substantially increase muscle-specific gene transcription. However, use of viral vector may limit the success of designed vector.

**Table 4: Location of cloned fragments in the constructed DES-LCR/MD1-EGFP vector**

| Fragment | Total Size | Components | Size (bp) | Location |
|----------|------------|------------|----------|----------|
| **Fragment 1** | 1069bp | DES-LCR Enhancer, Microdystrophin delR4-R23/delCT | 23 to 774 |
| | | P2A (2A peptide) | 775 to 1079 |
| | | EGFP (Enhanced Green Fluorescent Protein) | 3612 to 4697 |
| **Fragment 2** | 4396bp | P2A | 1069 to 4697 |
| | | EGFP (Enhanced Green Fluorescent Protein) | 4699 to 4755 |
| **Total** | | | 4762 to 5478 |

**Table 3: Characteristics of the designed DES-LCR/MD1-EGFP vector (8588bp)**

| BACKBONE | Vector backbone | pBluescript | Vector type | Mammalian expression |
|----------|----------------|-------------|-------------|----------------------|
| GROWTH IN BACTERIA | Bacterial resistance | Ampicillin | Growth temperature | 37°C |
| Copy number | High Copy |
| INSERT | DES-LCR enhancer and promoter sequences |
| Fragment 1 | MD1 and EGFP genes separated by P2A sequence |
| Species | Homo sapiens |
| Cloning method | Restriction and insertion (SnapGene) |

**Translational context:**
In this work, we designed a non-viral mammalian expression vector via computer-aided tools expressing transgenes under control of elements of LCR. DES-LCR/MD1-EGFP vector contains transgene MD1, a codon optimized and truncated version of dystrophin gene [12], under expression control of DES-LCR elements. Codon optimization by synonymous substitution is key to enhanced expression of recombinant protein in host cells [19]. P2A sequence in the vector links the MD1 gene to reporter gene EGFP. P2A is a self-cleaving peptide sequence, which has been extensively used for co-expression of multiple genes at a desired ratio in gene therapy and other biomedical research [20-21]. In silico analysis of DES-LCR/MD1-EGFP vector revealed that this vector could be used to express recombinant genes under control of elements of DES-LCR. It has been reported that enhancer and promoter region sequences of DES-LCR generated high level of expression comparable to highly active constitutive human cytomegalovirus (hCMV) promoter/enhancer [22], suggesting that DES-LCR constitute a promising control region, which can be used in expression vectors. Subsequently, elements of DES-LCR have been used in muscle-directed gene therapy viral vectors [18, 23]. DES-LCR/MD1-EGFP is a non-viral mammalian expression vector and hence a safer option over use of viral vectors expressing dystrophin gene for applications in muscle-directed gene therapy. Functional studies will be needed to validate the performance and usefulness of this vector in therapeutic settings. In recent past, novel designs of non-viral vector systems have been described with promising applications [24]. The designed vector in this work is another step towards tackling, potentially, the current challenges of vector design, which may prove to be useful in future, studies for treating multisystem diseases. Use of non-viral vectors comprising of human elements have been suggested ideal for human gene therapy as they deliver sustainable therapeutic levels of gene expression.
expression without adverse immunological effects [25]. Human LCRs are strong regulatory elements, hence, can be considered as preferred choice to construct additional regulatory systems for their applications in biomedical research. Ultimately, these vectors will inflate the traditional applications of gene therapy and will also lead towards newer other opportunities in the field of basic science and clinical research.

**Conclusion:**
We describe the design of non-viral vector with improved regulatory features using computer aided sequence analysis tools. This forms a framework towards design of new generation of gene therapy vectors with increased safety. It should be noted that this design should be validated with adequate experimental data.

**References:**
[1] Kotterman MA et al. Annual review of biomedical engineering. 2015 17:1. [PMID: 26643018]
[2] Mingozzi F & High KA. Blood 2013 122(1):23-36. [PMID: 23596044]
[3] Hardee CL et al. Genes 2017 8:2. [PMID: 28208635]
[4] Chandrasekaran AP et al. In Progress in molecular biology and translational science 2018 159:157-176. Academic Press. [PMID: 30340786]
[5] Corbo JC. Expert opinion on biological therapy 2008 8:5.
[6] Chira S et al. Oncotarget 2015 6:31. [PMID: 26362400]
[7] Trinh AT et al. Genetic vaccines and therapy 2009 7:13. [PMID: 20042112]
[8] Ye GJ et al. Human gene therapy 2015 27:1. [PMID: 26603570]
[9] Sharma BS et al. Journal of Computational Biology 2019 26:12. [PMID: 31305132]
[10] Gregorevic P et al. Nature Medicine 2006 12:7. [PMID: 16819550]
[11] Le Guiner C et al. Nature Communications 2017 8:16105. [PMID: 28742067]
[12] Foster H et al. Molecular Therapy 2008 16:11. [PMID: 18766174]
[13] Nyamay'Antu A et al. Cell & Gene Therapy Insights 2019 5:S1.
[14] Li Q et al. Blood 2002 100:9. [PMID: 12384402]
[15] Hanawa H et al. Blood 2004 104:8. [PMID: 15198957]
[16] Chendeb M et al. Oncotarget 2017 8:24. [PMID: 28415677]
[17] Chen H et al. In Suicide Gene Therapy 2019. Humana Press, New York, NY.
[18] Sarcar S et al. Nature Communications 2019 10:1. [PMID: 30700722]
[19] Gustafsson C et al. Trends in Biotechnology 2004 22:7. [PMID: 15245907]
[20] Ieda M et al. Cell 2010 142:3. [PMID: 20691899]
[21] Liu Z et al. Scientific Reports 2017 7:1. [PMID: 28526819]
[22] Zhang G et al. Human Gene Therapy 2004 15:8.
[23] Talbot GE et al. Molecular Therapy 2010 18:3. [PMID: 19935780]
[24] Megias R et al. International journal of pharmaceutics. 2017 518:270-80. [PMID: 28011343]
[25] Wong SP et al. In Prenatal Gene Therapy. 2012 891:133-167. Humana Press, Totowa, NJ.

**License statement:** This is an Open Access article which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. This is distributed under the terms of the Creative Commons Attribution License

**Edited by P Kangueane**

**Citation:** Sharma et al. Bioinformation 16(4): 307-313 (2020)
Articles published in BIOINFORMATION are open for relevant post publication comments and criticisms, which will be published immediately linking to the original article for FREE of cost without open access charges. Comments should be concise, coherent and critical in less than 1000 words.
