Novel method to load a mammalian artificial chromosome (MAC) with multiple genes

Ph. D. thesis summary

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2014.

Szeged

Introduction

Recently, the field of gene therapy is rapidly developing in clinical applications and it draws public attention, but it is still a controversial issue. Gene therapy intervenes in cellular processes on the molecular level by modifying its genome with newly introduced genetic information. The replacement or the correction of deficient genes could be carried out both in somatic and germ cells, but it is only permitted in somatic cells. New information could be introduced into cells by using carriers/vectors, and they could be delivered by infection, transfection reagents (e.g. liposomes, polimers) or physical methods (e.g. electroporation). Nowadays, the most often applied carriers are genetically modified viruses (e.g. retroviruses, lentiviruses, adenoviruses). In this case, gene therapy could cause mutations and serious immune response in treated patients. As far as we know, presently there is no proper gene carrier, which meets the strict requirements of an ideal vector (e.g. it shouldn't integrate into the host genome and it should have an excessive transgene carrying capacity).

Therefore, mammalian artificial chromosomes may open a new era, as novel gene therapy vectors. Contrary to virus-based vectors mammalian artificial chromosomes have an excessive transgene carrying capacity and they don't integrate into the host genome. Recently, a Platform Artificial Chromosome Expression System (Platform ACEs) was developed that is based on Satellite DNA-based Artificial Chromosomes (SATACs). Transgene(s) can be loaded onto the Platform ACEs by using a modified lambda integrase (ACE integrase), which has numerous acceptor sites on the Platform ACE. Therefore, it is possible to load several transgenes onto the Platform ACEs.

We combined mammalian artificial chromosomes and stem cells in a therapeutic approach to treat the mouse model of the recessive lethal, monogenic Krabbe disease. This preclinical experiment was fairly successful, since treated mutant mice lived up to 4 times longer than the untreated

animals. Moreover, it is getting more and more necessary to develop a gene loading vector, which is suitable to treat complex genetic diseases and cancers. The Platform ACE system is theoretically suitable for this task. However, there is a major inherent problem in the present system, since it is necessary to use a new antibiotic resistance gene for each transgene-loading onto this chromosome. Moreover, the number of presently available antibiotic marker genes is limited and their overexpression could lead to undesired side-effects.

Aims of the study

During our experiments, we aimed to develop a new gene loading system, which enables the loading of Platform ACEs with numerous genes by using only one selection marker gene cassette. We named this novel plasmid vector as superloading vector or shortly pST.

We aimed to load two well-detectable transgenes in pST vectors onto the Platform ACEs in the Y29-13D-SFS *Chinese hamster* ovary (CHO) cell line. One of these constructs carries the mCherry protein coding gene, and the other carries the beta-galactosidase gene (LacZ). In both cases, we planned to carry out the transfections together with the ACE integrase expressing plasmid, and we aimed to verify the stable presence of superloading vectors in the cells by G418 selection. We wanted to prove site-specific integration by using polymerase chain reactions carried out with specific primer pairs on the genomic DNA samples derived from the selected cell lines. We aimed to examine the cells, which show red fluorescence by fluorescent microscopy. We wanted to reveal the presence of transgenes on the Platform ACEs in the selected cell lines by FISH experiments. At the end of the first superloading cycle, we planned to remove the whole selection marker gene expression cassette from the cells by the transient expression of the Cre recombinase. The plasmid, which expressed this enzyme, was introduced into the selected cell line by transfection. We planned to establish cell lines, in which the antibiotic selection marker gene cassette is completely removed from the Platform ACEs, by Ganciclovir selection. In the second superloading cycle, aside from the above mentioned methods, we wanted to use LacZ staining for selection of the appropiate cell lines.

Methods

1. Production of transgene carrying plasmids (plasmid DNA purification, restriction endonuclease treatment, DNA ligation reactions, agarose gel electrophoresis, DNA fragment purification, transformation of bacteria and growth on selective plate, DNA sequence design and alignment with software).
2. Maintenance and transfection of mammalian cell cultures.
3. Genomic DNA purification from the selected cell lines.
4. Polymerase chain reaction (PCR).
5. Fluorescent in situ hybridization (preparation of chromosomes in metaphase stage from the selected cell lines, DNA-DNA hybridization, immunodetection).
6. Fluorescent microscopy.
7. LacZ staining in cell cultures.

Results and discussion

A vector system was earlier developed for gene loading onto the Platform ACE system. The first element of this system is an “entry” vector, which contains an expression cassette including a multi-cloning site (MCS) for the insertion of a transgene. This expression cassette consists of a CMVIE-chicken beta-actin-beta-globin hybrid promoter and an SV40 polyA signal. The whole expression cassette can be moved from the “entry” vector into the Platform ACEs-targeting ATV vectors by using the I-Ceu and PI-PspI yeast homing endonucleases, because the ATVs are also carrying the recognition sites of these enzymes.

In the experiments described here, we developed the pST ATV plasmid, which contains a promoterless neomycin antibiotic resistance gene and a HSV-TK (*Herpes simplex* thymidine kinase) expression cassette flanked by LoxP sites in the same orientation. We loaded two, well-detectable plasmid constructs onto a Platform ACEs in a *Chinese hamster* ovary (CHO) cell line. The first superloading plasmid carried the mCherry gene. The pST vector together with its cargo is loaded onto the Platform ACEs by the ACE integrase. This enzyme fulfills the loading event by performing a site-specific recombination between the attP (on the Platform ACEs) and the attB sites (on the pST ATV). The ACE integrase is a modified lambda integrase. The modification renders the integrase functionally independent of bacterial host cell factors and capable of operating in a mammalian context. The ACE integrase is transiently expressed from the pCXLamIntROK plasmid, which is co-transfected with the pST ATV vector into the target cells. After the loading procedure, the promoterless neomycin resistance gene replaces the puromycin resistance gene on the Platform ACEs and acquires its promoter. Therefore, the transgene carrying cell lines become G418-resistant. In this way, we successfully produced red fluorescent protein expressing cell lines. We checked these established cell lines by using PCR reactions specific to the integration event, FISH

experiments and fluorescence microscopy. At the end of the first superloading cycle, we removed the whole neomycin-HSV-TK expression cassette from the cells by the transient expression of the Cre recombinase. Therefore, the ACEs was able to receive a new transgene, without the application of a new selection marker gene. In the second superloading cycle, we loaded the LacZ containing pST plasmid onto the Platform ACEs, which has already carried the mCherry. We successfully induced site-specific integration event again. We selected the cell lines, which were carrying both the mCherry and the LacZ genes on the Platform ACE, by using PCR rections specific to the integration event, FISH experiments, fluorescent microscopy and LacZ staining.

The application of this superloading system was very easy and effective. Moreover, this procedure presents no serious toxicity to the cells. We conclude that it is possible to remove all the selectable marker genes in the last step before therapeutic applications. This reduces the risk of side-effects due to the expression of antibiotic resistance coding genes.

Acknowledgements

I would like to thank my supervisor **Dr. Róbert Katona**, that he allowed me to do my PhD work in his group. With his comprehensive specialized knowledge of biology he was very helpful both in the labwork and in the preparation of papers. I had the ability to count on his knowledge in professional matters.

I am grateful to **Prof. Dr. Gyula Hadlaczky** for his contribution to my PhD work in this group. He followed my experiments patiently and also criticized when it was necessary.

Here I want to thank **Dr. Imre Cserpán** for letting me study the bases of molecular biology during my last graduate years.

Prof. Dr. Andor Udvardy kept also an eye on my PhD work and he always tried to help my experiments with useful advices. I owe him thanks for his conscientious attitude and his unlimited patience.

I would also like to express my gratitude towards my past and present colleagues **Dr. Gábor Péter Blazsó**, **Dr. Erika Csonka**, **Dr. Katalin Fodor**, **Dr. Tünde Praznovszky** and **Dr. Vilmos Tubak** because I could count on their knowledge in either professional or non professional matters anytime.

I would like to thank the other members of our research group **Mária Deák**, **Csilla Horváth**, **Kinga Katonáné Székely-Szűcs, Judit Kereső**, **Tímea Kovács**, **Gyöngyi Mózesné Holló**, **Balázs Odrovics** and **Márta Rózsavölgyi** for their contribution to my experiments.

Here I would like to be grateful to all my **colleagues at the Institute of Genetics, BRC**, that I was able to work in a supportive environment.

I want to thank **my parents**, **my sister** and **my boyfriend** for their direct or indirect help in my professional development.

Publications

Papers:

- Toth, A., Fodor, K., Praznovszky, T., Tubak, V., Udvardy, A., Hadlaczky, Gy., Katona, R. L. Novel method to load multiple genes onto a mammalian artificial chromosome. *Plos One,* 2014 Jan 15;9(1):e85565. doi: 10.1371/journal.pone.0085565. eCollection 2014 Jan 15.
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