Biochemical and Biophysical Research Communications 442 (2013) 44–50

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Highly stable maintenance of a mouse artificial chromosome in human cells and mice

Kanako Kazuki [a,b], Shoko Takehara [a,b], Narumi Uno [a], Natsuko Imaoka [a,b], Satoshi Abe [a], Masato Takiguchi [a], Kei Hiramatsu [a], Mitsuo Oshimura [a,b,c,*], Yasuhiro Kazuki [a,b,*]

[a] Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan
[b] Chromosome Engineering Research Center, Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan
[c] JST, CREST, 5 Sanbon-cho, Chiyoda-ku, Tokyo 102-0075, Japan

ARTICLE INFO

Article history:
Received 25 October 2013
Available online 9 November 2013

Keywords:
Mouse artificial chromosome
Microcell-mediated chromosome transfer
Transchromosomic mouse
Gene delivery

ABSTRACT

Human artificial chromosomes (HACs) and mouse artificial chromosomes (MACs) display several advantages as gene delivery vectors, such as stable episomal maintenance that avoids insertional mutations and the ability to carry large gene inserts including the regulatory elements. Previously, we showed that a MAC vector developed from a natural mouse chromosome by chromosome engineering was more stably maintained in adult tissues and hematopoietic cells in mice than HAC vectors. In this study, to expand the utility for a gene delivery vector in human cells and mice, we investigated the long-term stability of the MACs in cultured human cells and transchromosomic mice. We also investigated the chromosomal copy number-dependent expression of genes on the MACs in mice. The MAC was stably maintained in human HT1080 cells in vitro during long-term culture. The MAC was stably maintained at least to the F8 and F4 generations in ICR and C57BL/6 backgrounds, respectively. The MAC was also stably maintained in hematopoietic cells and tissues derived from old mice. Transchromosomic mice containing two or four copies of the MAC were generated by breeding. The DNA contents were comparable to the copy number of the MACs in each tissue examined, and the expression of the *EGFP* gene on the MAC was dependent on the chromosomal copy number. Therefore, the MAC vector may be useful not only for gene delivery in mammalian cells but also for animal transgenesis.

1. Introduction

The introduction of large genes or gene clusters into mice allows the correct expression of transgenes by including essential remote regulatory elements [1]. Human chromosome fragments (hCFs) derived from normal fibroblasts were used as a vector for animal transgenesis, including the introduction of Mb-sized large genomic inserts into mice via microcell-mediated chromosome transfer (MMCT) technology [2,3]. Double transchromosomic (Tc) mice containing two individual hCFs carrying *IgH* and *Igk* produced antigen-specific human antibodies [4]. However, the mitotic stability of hCFs in mice varies, and large hCFs cannot be transmitted through the germline [2,5,6]. Cloning the desired genomic region into the mitotically stable hCF allowed us to generate Tc mice containing multiple large genomic inserts, which could not be cloned using conventional vectors such as plasmids and bacterial artificial chromosomes (BACs) [5,7]. However, hCFs contain several structurally undefined regions with many endogenous genes, which cause partial trisomy in cells propagating these hCFs. This may affect the physiological gene expression and the normal development. To overcome this, several groups engineered human artificial chromosomes (HACs) by random segmentation or targeted telomere-associated chromosomal fragmentation in homologous recombination-proficient chicken DT40 cells [5,8–10]. However, germline transmittable Tc mice containing multiple copies of HACs with Mb-sized large inserts have never been generated, possibly because of the instability of HACs in germ cells. Although hCFs and HACs containing large regions of genomic DNA can be autonomously maintained in Tc mice, their retention rate varies [3,6,11–14]. Thus, we constructed novel mouse artificial chromosome (MAC) vectors from a native mouse chromosome by chromosome engineering to improve the retention rate [15]. Previously, a MAC vector containing the *EGFP* gene, which can be used to monitor the cells, was used to determine its stability in vivo. The stability of this MAC in mouse tissues and hematopoietic cells was higher than that of other reported mammalian artificial

* Corresponding authors at: Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan. Fax: +81 859 38 6210.
E-mail addresses: oshimura@grape.med.tottori-u.ac.jp (M. Oshimura), kazuki@grape.med.tottori-u.ac.jp (Y. Kazuki).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.10.171

chromosomes including hCFs, HACs, and murine satellite DNA-based artificial chromosomes (mSATACs) [16]. The stability and germline transmission (GT) efficiency of hCFs, HACs, and mSATACs differed with genetic background, generation, age, and sex [4,6,12,14,17,18]. MACs will be a powerful tool to generate Tc mice carrying multiple Mb-sized genes for humanized animal models if they have high stability and GT efficiency and if multiple copies can be introduced into mice. Furthermore, if the MAC is stable in human cells, the same MAC containing a desired gene may be used for functional analysis in both mice and human cells. Therefore, we investigated: (i) MAC stability in HT1080 cells, (ii) MAC stability and GT ratio in different genetic backgrounds and sexes, (iii) MAC stability in aged mice, (iv) MAC copy number per cell in tissues, and (v) MAC copy number-dependent gene expression in tissues.

2. Materials and methods

2.1. Cell culture

HT1080 were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) plus 10% fetal bovine serum (FBS). Chinese hamster ovary (CHO) cells containing MAC1 were constructed as previously described [15]. The CHO (MAC1) cells were maintained in Ham's F-12 nutrient mixture (Invitrogen, Carlsbad, CA, USA) plus 10% FBS with 800 μg/mL G418 (Promega, Tokyo, Japan).

2.2. MMCT

MMCT was performed as described previously [2,19]. CHO cells containing MAC1 were used as donor microcell hybrids. The structure of MAC1 which consists of a centromere from mouse chromosome 11, *EGFP* flanked by HS4 insulators, *PGKneo*, 3′HPRT-loxP site, *PGKpuro* and telomeres, was described in detail previously [15]. Briefly, HT1080 cells were fused with microcells prepared from donor hybrid CHO (MAC1) cells and selected with G418 (600 μg/mL). In each line, MAC1 was characterized by polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH) analyses.

2.3. Quantitative genomic PCR analyses

Genomic DNA was extracted from Tc mouse tissues using a genomic DNA extraction kit (Gentra System, Minneapolis, MN, USA). Quantitative genomic PCR analyses detected the *EGFP* gene on MAC1 using the Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) and 2 × FastStart Universal SYBR Green Master (Rox) (Roche Applied Science). Brain DNA of MAC1 Tc mice was used as a positive control for normalization, while DNA from C57BL/6 mouse tissues was used as a negative control. An endogenous mouse gene, *Ravor2*, was used as an internal standard (Takara, Shiga, Japan). The following primers were used to amplify the *EGFP* gene: 5′-tctatatcatggccgacaagc-3′ and 5′-gttgtggcggatcttgaagt-3′.

2.4. Cytogenetic analyses

Slides of testis or cultured lymphocytes derived from MAC1 Tc mice were stained with quinacrine mustard and Hoechst 33258 or Giemsa stain to enumerate chromosomes. Images were captured using an AxioImagerZ2 fluorescence microscope (Carl Zeiss GmbH). FISH analyses were performed using fixed metaphase or interphase spreads of HT1080 cell hybrids using biotin-labeled (Roche, Basel, Switzerland) mouse COT-1 DNA (Invitrogen) essentially as described previously [2]. Chromosomal DNA was counterstained with DAPI (Sigma). Images were captured using the NIS-Elements system (Nikon, Tokyo, Japan).

2.5. Quantitative reverse transcription (RT)-PCR analyses

Total RNA from Tc mouse tissue specimens was prepared using ISOGEN (Nippon Gene, Tokyo, Japan), purified using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and treated with RNase-free DNase I (Wako). First-strand cDNA synthesis was performed using random hexamers and SuperScript III reverse transcriptase (Invitrogen). *β-Actin* was used as the internal control. Brain cDNA of MAC1 Tc mice was used as a positive control for normalization, while cDNA from C57BL/6 mouse tissues was used as a negative control. Quantitative RT-PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR system and 2 × EXPRESS qPCR Supermix with Premixed ROX (Life Technologies). The following primers were used: *β-actin*, 5′-ggatgcagaaggagattactgc-3′ and 5′-ccaccgatccacacagagta-3′; and *EGFP*, 5′-cctgaagttcatctgcacca-3′ and 5′-ggtcagggtggtcacgag-3′. TaqMan probe #63 and #37 were used to detect *β-actin* and *EGFP*, respectively.

2.6. Breeding analyses

Jcl:ICR (ICR) and C57BL/6JJcl (B6) mice were purchased from Japan Crea. Previously, we generated F1 mice by mating chimeric female mice with ICR males [15]. Briefly, F1 mice were bred with either ICR or B6 mice. In the ICR background, the mice were backcrossed with ICR until the F8 generation. In the B6 background, the mice were backcrossed with B6 until the F4 generation. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University.

3. Results and discussion

3.1. Stability of MAC1 in HT1080 cells

We used the MAC1 vector with *EGFP* to monitor the gene expression and stability of the MAC. To investigate the stability of a MAC in human cells, MAC1 was transferred to the human fibrosarcoma cell line HT1080 by MMCT. Three GFP-positive clones were selected and examined. FISH analyses showed that MAC1 was present as an individual chromosome in the HT1080 cells (Fig. 1A). After the HT1080 (MAC1) cells were cultured for about 3 months with or without selection, the stability of the MAC1 and GFP expression on the MAC1 were tested. FISH analyses revealed that the MAC1 was independently and stably maintained in HT1080 cells (>90%) (Fig. 1B), and most of the HT1080 (MAC1) cells were GFP-positive (Fig. 1C). The loss of MAC1 (<10%) in FISH analyses was comparable to the ratio of the GFP-negative cells. This suggests that MAC1 is maintained stably and that the *EGFP* gene on MAC1 is expressed stably even after long-term culture in vitro without antibiotic selection. Previously, an mSATAC was created via amplicon-dependent de novo chromosome formation induced by integrating exogenous DNA sequences into centromeric/rDNA regions near the pericentric heterochromatin or acrocentric chromosomes. The mSATAC consisting of murine pericentric satellite DNA was not stable in a human cell line, but it was relatively stable in a mouse cell line [20]. Because MAC1 was stable in human cells and mouse embryonic stem (ES) cells, a mouse chromosome or MAC with a natural centromere structure may be important for stability in human and mouse cells.