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Effects of palindrome structure on *Dhfr* amplification in Chinese hamster ovary cells

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ABSTRACT

Chinese hamster ovary (CHO) cells are widely used in producing therapeutic proteins. Gene amplification techniques are frequently used in improving protein production, and the dihydrofolate reductase (DHFR) gene amplification system is most widely used for the CHO cell line. We previously constructed a CHO genomic bacterial artificial chromosome (BAC) library from a mouse *Dhfr*-amplified CHO DR1000L-4N cell line and found one BAC clone (Cg0031N14) containing a CHO genomic DNA sequence adjacent to *Dhfr*. The BAC clone contained a large palindrome structure with a small inverted repeat in the junction region. To investigate the effect of the palindrome structure derived from the BAC clone Cg0031N14 on *Dhfr* amplification in CHO cells, we constructed plasmids that contain part or the whole junction region of the palindrome structure. The transfected CHO DG44 cells containing part or the whole junction region of the palindrome structure could adapt quickly to high methotrexate (MTX) concentrations. Moreover, the cells containing the whole junction region of the palindrome structure showed a high ratio of GFP-positive cells during gene amplification. On the basis of these results, we estimated that the junction region plays an important role in gene amplification in CHO cells.

1. Introduction

Mammalian cells have been widely used in producing recombinant therapeutic proteins because of their correct post-translational modifications [1–3]. However, the productivity of recombinant mammalian cells is much lower than that of microbial cells [1,4]. To overcome this disadvantage, various approaches, i.e., cell line improvement [5–7], cell culture medium modification [8,9], and the development of effective scale-up processes and environmental conditions [10–12], were carried out in mammalian cell systems. Gene amplification techniques are frequently used for the high-level production of foreign proteins in recombinant mammalian cell technology. Chinese hamster ovary (CHO) cells, particularly dihydrofolate reductase (DHFR)-deficient DG44 cell lines, are widely used for the commercial-scale production of therapeutic proteins as a host cell for gene amplification. DHFR gene amplification is frequently used in constructing productive CHO cells, resulting in high productivities as well as high gene copy numbers [13]. We have established several mouse *Dhfr*-amplified CHO cell lines and elucidated the relationships between the productivity and stability of gene-amplified cells and the location of the amplified gene [14].

Previously, we analyzed the structure of amplicons by screening a constructed CHO genomic bacterial artificial chromosome (BAC) library from a mouse *Dhfr*-amplified CHO DR1000L-4N cell line. The determined sequence had a palindrome structure containing a small inverted repeat in the junction region [15]. We speculated that the obtained palindrome structure plays an important role in exogenous *Dhfr* amplification in the CHO cell line.

In this study, to determine the effect of the palindrome structure on *Dhfr* amplification in CHO cells, we constructed three types of expression vectors with or without the junction region of the proposed amplicon. Moreover, we investigated the gene amplification and expression levels in transfected CHO DG44 cells.

2. Materials and methods

2.1. Cell line and medium

The CHO DG44 cell line (dhfr$^-$), which was kindly provided by Dr. L. Chasin of Columbia University, was used as the host cell line. The host cells were maintained in Iscove's modified Dulbecco's medium (IMDM) (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (JRH Biosciences, Kansas, USA), hypoxanthine (13.6 mg/L), and thymidine (2.42 mg/L). Transfected CHO cells (dhfr$^+$; derived from DG44) were cultivated in IMDM containing 10% dialyzed FBS (without hypoxanthine and thymidine) and methotrexate (MTX) (Sigma) at various concentrations. During gene amplification, the transfected cells were cultivated with 50, 100, 250,

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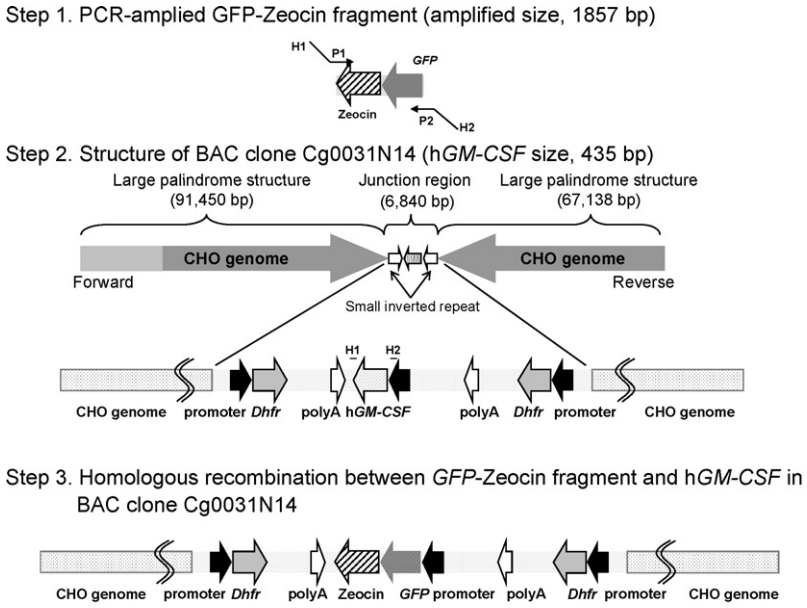

Fig. 1. Construction of BAC clone Cg0031N14–GFP by homologous recombination. H1 and H2 refer to homology extensions or regions. P1 and P2 refer to priming sites.

and 500 nM MTX concentrations. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Construction of expression plasmids

The structure of the *Dhfr* amplicon in the BAC clone Cg0031N14 derived from the gene-amplified CHO DR1000L-4N cells was determined previously [15]. The structure has a large palindrome structure containing a small inverted repeat in the junction region. This small inverted repeat originates from the integrated vector. On the basis of this junction region, the expression vectors were constructed. The human granulocyte-macrophage colony stimulating factor gene (h*GM-CSF*) in the junction region in the BAC clone Cg0031N14 was replaced with the green fluorescent protein gene (*GFP*) using a modified method described previously [16,17] (Fig. 1). In brief, the BAC clone Cg0031N14 was extracted using a Qiagen large-construction kit (Qiagen, Maryland, USA). To change h*GM-CSF* to *GFP*, a GFP–zeocin fragment was constructed by two polymerase chain reactions (PCRs). GFP and zeocin fragments were obtained by the 1st PCR using EGFP (Clontech, Mountain View, CA, USA) and pZeoSV2(+) plasmids (Invitrogen, Carlsbad, CA, USA) as the templates, respectively. A GFP–zeocin fragment was constructed by the 2nd PCR using the 1st PCR products as the template and the primers P1 (5′-TCAGTCCTGCTCCTCGGCCA-3′) and P2 (5′-ATGGTGAGCAAGGGCGAGGA-3′), with the 50-bp arms H1 (5′-GTTTCAT GAGA-GAGCAGCTCCCCGGCTTGGCCAGCCTCATCTGGCCGGTC-3′) and H2 (5′-GGGGGG-GGGGGGGGGGGACACAGAGAGAAAGGCTAAAGTTCTCTGGAGG-3′) homologous to the BAC sequence (Step 1). *Escherichia coli* BW25113, which was kindly provided by Professor H. Mori (Nara Institute of Science and Technology, Japan) and was carrying the BAC clone Cg0031N14 and pKD46 (λ Red recombination system), was grown in SOB culture with chloramphenicol (12.5 μg/ml), ampicillin (100 μg/ml), and L-arabinose (150 μg/ml) at 30 °C to an OD_{600} of ~0.6. Electrocompetent *E. coli* BW25113 was transformed with the GFP–zeocin gene-containing PCR construct. Cm^R/$Zeocin^R$ transformants were screened and their sequence was determined to confirm the desired homologous recombination (Step 3). Positive colonies were grown overnight at 42 °C to remove pKD46, and then pKD46 removal was confirmed by an ampicillin sensitivity test.

pSV2-dhfr/GFP, pcD-core region and pcD-repeat free core region plasmids were derived from the BAC clone Cg0031N14–GFP (Fig. 2). The pSV2-dhfr/GFP vector (vector A) was constructed from pSV2-dhfr/hGM-CSF [13] and fragment a–a′ of the BAC clone Cg0031N14–GFP. The pcD-core region (vector B) was constructed from pcD-hGM-CSF (ATCC 57594) and the core region (junction region containing two *Dhfr* copies and one *GFP*, which is fragment b–b′ in the BAC clone Cg0031N14–GFP). The pcD-repeat free core region vector (vector C) was constructed from pcD-hGM-CSF and the repeat free core region (part of the junction region containing one *Dhfr* and one *GFP*, which is fragment c-c′ in the BAC clone Cg0031N14–GFP).

2.3. Transfection and gene amplification

The constructed plasmids were purified using a QIAGEN plasmid Midi kit (Qiagen) and transfected into the CHO DG44 cells using Lipofectamine PLUS (Invitrogen). The plasmids were linearized by digesting with a restriction enzyme that cuts at a single site prior to the transfection. The pSV2-dhfr/GFP (vector A) was digested by *Apa*I, and the pcD-core region (vector B) and pcD-repeat free core region (vector C) were digested by *Pvu*I. The cells were inoculated into 10-cm dishes and cultivated for 48 h using IMDM without hypoxanthine and thymidine. The cells were cultivated until semiconfluent (from 0.8 to 1.0 × 10^6 cells/ml) and passaged three times before adding MTX. In the *Dhfr*-amplification step, the transfected cells were cultivated in IMDM without hypoxanthine and thymidine but with MTX at various concentrations of 50, 100, 250 and 500 nM.

2.4. Analysis of cell growth and MTX adaptation time

Cell growth in the *Dhf* amplification step was evaluated using batch-replica cultivation using the transfected cells. The cells were inoculated into a 24-well plate at an initial concentration of 2 × 10^4 cells/ml. The cells were harvested daily from each two wells, and cell concentration was determined using a hemacytometer (ERMA, Tokyo, Japan). MTX adaptation time was evaluated from cultivation time. The inoculated cells were cultivated until semiconfluent (0.8–1.0 × 10^6 cells/ml). The cells were harvested and resuspended in a fresh medium at a concentration of 2 × 10^4 cells/ml. MTX adaptation time was defined as the period from inoculation to semiconfluent. We performed three passages to evaluate MTX adaptation. The specific growth rate based on the number of viable cells was calculated as described previously [18].

2.5. Slot-blot hybridization

Slot-blot hybridization was used to determine the copy number of *GFP* as described previously [19]. Briefly, the samples were serially diluted with TE buffer (1 mM Tris–HCl and 0.5 mM EDTA), boiled in a denaturing solution (0.4 M NaOH and 10 mM EDTA) for 10 min, and neutralized by adding 2 M ammonium acetate solution. The neutralized samples were loaded into each slot and capillary-transferred to a positively charged Hybond-N^+ nylon membrane using an assembled Bio-Slot SF apparatus (Bio-Rad, Hercules, CA, USA). The blotted membrane was rinsed in 2× SSC for 10 min, dried, and cross-linked twice at 1,200 × 100 μJ. Probe labeling and hybridization signal detection were performed by following the instructions in the DIG DNA labeling kit (Roche Applied Science). The copy number of *GFP* was estimated by comparing the sample slots with the slots produced from a dilution series of standard *GFP* using ImageQuantTL (Fuji Film, Tokyo, Japan). A Quant-iT™ PicoGreen® dsDNA reagent and kits (Invitrogen) were used for *GFP* quantitation.

2.6. Flow cytometry

Flow cytometry was performed on a Beckman–Coulter EPICS-XL-MCL flow cytometer (Beckman, Fullerton, CA, USA) equipped with a 15-mW air-cooled argon ion laser-emitting light at 488 nm. The cells were cultivated in IMDM with 10% dialyzed FBS containing MTX for 3–4 days. The medium was removed, and the cells were washed with PBS, treated with 0.025% trypsin in PBS for about 5 min at 37 °C and collected by centrifugation at 653 × *g*. The cell pellet was resuspended in PBS at a concentration of about 10^6 cells/ml. Acquisition and analysis were performed using Coulter XL system II; 5000 events were acquired.