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Identification and analysis of specific chromosomal region adjacent to exogenous *Dhfr*-amplified region in Chinese hamster ovary cell genome

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Chinese hamster ovary (CHO) cells are widely used for the stable production of recombinant proteins. Gene amplification techniques are frequently used to improve of protein production, and the dihydrofolate reductase (DHFR) gene amplification system is most widely used in 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 one BAC clone (Cg0031N14) containing the CHO genomic DNA sequence adjacent to *Dhfr* was selected. To identify the specific chromosomal region adjacent to the exogenous *Dhfr*-amplified region in the CHO cell genome, we performed further screening of BAC clones to obtain other *Dhfr*-amplified regions in the CHO genome. From the screening by high-density replica filter hybridization using a digoxigenin-labeled pSV2-dhfr/hGM-CSF probe, we obtained 8 new BAC clones containing a *Dhfr*-amplified region. To define the structures of the 8 BAC clones, Southern blot analysis, BAC end sequencing and fluorescence *in situ* hybridization (FISH) were performed. These results revealed that all the selected BAC clones contained a large palindrome structure with a small inverted repeat in the junction region. This suggests that the obtained amplicon structure in the *Dhfr*-amplified region in the CHO genome plays an important role in exogenous gene amplification.

[**Key words:** Bacterial artificial chromosome library; Chinese hamster ovary cell; Gene amplification; Dihydrofolate reductase (DHFR); Genome; Palindrome structure]

The Chinese hamster ovary (CHO) cell line is one of the first recombinant mammalian cell lines successfully developed for use in the industrial production of therapeutic proteins. CHO cells are now considered the mammalian equivalent of *Escherichia coli* cells in research and biotechnology. With a recent increase in the demand for therapeutic proteins, there is a requirement for greater biomanufacturing capacity. To overcome this requirement, various approaches, i.e., cell line improvement (1, 2), cell culture medium modification (3), improvement of product quality including glycosylation (4), and development of effective scale-up process and environmental conditions (5–7) were carried out in CHO cell systems.

To attain high productivity of a recombinant protein in mammalian cells, attention has been paid to the development of gene-amplified cell lines with a high specific rate of protein production (1, 8). Dihydrofolate reductase (DHFR)-deficient CHO cells and the amplifiable selectable marker *DHFR* are routinely used to establish cell lines that produce clinically useful amounts of products (1, 9). Establishing CHO cell lines that maintain the stability of protein production is of utmost importance, particularly for industrial companies that eventually hope to market proteins produced by such cell lines. CHO cell lines have a very unstable karyotype owing to their chromosomal rearrangements arising from translocations and homologous recombination, particularly as a result of amplification procedures (10). Ruiz and Wahl reported that amplification in a CHO cell line begins with the production of unstable extrachromosomal structures, and their subsequent integration initiates a wave of chromosomal instability. Many studies have shown that large DNA palindromes can be generated by short inverted repeats with an adjacent DNA double-strand break in a CHO cell line (11–13), and that large DNA palindrome regions initiate iterative cycles of genome instability, leading to a wide variety of chromosomal aberrations (14).

Recently, we have established several mouse *Dhfr*-amplified CHO cell lines that are more stable and productive than any other cell lines and clarified the relationships between the productivity and stability of gene-amplified cells and the location of the amplified gene (15). The *DHFR* amplicon and its locus have been defined in the endogenous CHO *DHFR*-amplified CHOC 400 cell line (16, 17). The head-to-head *DHFR* amplicon in CHOC 400 has been used as a model amplicon for studies of gene amplification, and its structure generally supports the sister chromatid model of gene amplification (18, 19). However, the genomic structure of the exogenous *Dhfr* amplicon in the gene-amplified CHO remains to be elucidated.

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In our previous study, we constructed a CHO genomic bacterial artificial chromosome (BAC) library from a mouse *Dhfr*-amplified CHO DR1000L-4N cell line for the genome-wide analysis of CHO cell lines. A BAC library, which represents the entire genome of an organism, can reduce the complexity of the genome and provide clones physically separated in an addressable format (20, 21). BAC libraries also provide scaffolding for mapping sequence contigs to localized genomic regions using a direct genomic shotgun sequencing approach. Our BAC system is highly stable, capable of cloning large inserts, and useful for constructing a total genomic library with high stability (20, 21). The constructed CHO genomic BAC library was estimated to cover five times the CHO genome size (21).

Here, we report the further screening to obtain the specific chromosomal region adjacent to the exogenous *Dhfr*-amplified region from the CHO genomic BAC library. Information on the structure of the cloned DNA region that contains the exogenous *Dhfr* is important for our understanding of the mechanism underlying *Dhfr* amplification and the stability of recombinant protein expression in this system.

MATERIALS AND METHODS

Cell cultures The Chinese hamster ovary DR1000L-4N cell line was used. This cell line was established from the CHO DG44 cell line by stepwise methotrexate (MTX) selection and contains 173 and 166 copies of mouse *Dhfr* and the human granulocyte–macrophage colony stimulating factor gene (h*GM-CSF*) determined by slot blot hybridization, respectively (15, 22). Cells were cultivated in Iscove's modified Dulbecco's medium (Sigma, St. Louis, MO, USA) containing 10% dialyzed fetal bovine serum (JRH Biosciences, Kansas, USA) without hypoxanthine and thymidine at 37 °C in a humidified atmosphere containing 5% CO_2. Details of the other procedures were previously described (15).

Isolation of BAC clones carrying amplified chromosomal region in a CHO genomic BAC library To isolate BAC clones carrying a *Dhfr*-amplified chromosomal region, probe hybridization to high-density replica (HDR) filters was performed as described previously (20, 21). Briefly, twenty-two HDR filters were developed using BioGrid BG600 (Biorobotics, Dorchester, Dorset, UK) and 18,452 BAC clones were blotted onto each nylon membrane filter (22 cm × 22 cm). We used digoxigenin (DIG)-labeled pSV2-dhfr/hGM-CSF (21) as a probe to identify positive clones by HDR-filter hybridization. DIG-labeling of the probe was performed with a DIG DNA labeling and detection kit (Roche Applied Science, Manheim, Germany). The hybridized-HDR filter was washed twice with a low-stringency solution (2× SSC and 0.1% SDS) for 10 min at room temperature and washed twice with a high-stringency solution (0.2× SSC and 0.1% SDS) for 10 min at 65 °C. The labeled probe was detected using an alkaline phosphatase (AP)-labeled anti-DIG antibody and CSPD, disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′- (5′-chloro)-tricyclo (3.3.1^{3,7}) decan}-4-yl)-1-phenyl-phosphate (Roche Applied Science) as a substrate of AP. The membrane was exposed to an X-ray film (Kodak) and developed. Southern blot analysis using mouse *Dhfr* or h*GM-CSF* as a probe was carried out to confirm positive BAC clones. BAC clones containing *Dhfr* identified by HDR-filter hybridization were digested individually (single digestion) with each of the restriction enzymes (*Bam*HI, *Eco*RI and *Hin*dIII) and fractioned by 1% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate/1 mM EDTA, pH 8). The separated DNA was transferred to a Hybond-N$^+$ nylon membrane (Amersham Bioscience, Halfont, England). The membrane was hybridized with a mouse *Dhfr*- or h*GM-CSF*-specific probe labeled with DIG-dUTP. DIG labeling of this probe DNA was performed by the random priming method (Roche Applied Science) using PCR analysis. The mouse *Dhfr* and human *GM-CSF* cDNA probes were amplified by PCR with the primers 5′-AGCTTTATCCCCGCTGCCATCATGG-3′ and 5′-CCAATTATGTCACACCACAGAA-GTAAGG-3′ (the amplified DNA size, 708 bp), and 5′-GGCTGCAGAGCCTGCTGCTCTTG-3′ and 5′-TCCTGGACTGGCTCCCAGCAGTC-3′ (the amplified DNA size, 426 bp), respectively. Probe labeling and the detection of hybridization signals were performed by following the instructions in the DIG high prime DNA labeling and detection kit (Roche Applied Science). Finally, the membrane was exposed to an X-ray film (Kodak) and developed. The insert sizes of the isolated BAC clones were determined by pulsed field gel electrophoresis (PFGE) (CHEFF-DR III system, Bio-Rad, Hercules, CA, USA) on 1.0% agarose gel after digestion with *Not*I.

Quantification of gene copy number The slot-blot hybridization was used to determine the copy number ratio of h*GM-CSF* to *Dhfr* by modified methods described previously (23). Briefly, samples were serially diluted with TE buffer (1 mM Tris–HCl and 0.5 mM EDTA) and boiled in a denaturing solution (0.4 M NaOH and 10 mM EDTA) for 10 min, and then neutralized with 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). The blotted membrane was rinsed in 2× SSC for 10 min, dried, and cross-linked twice at 1200 × 100 μJ. The labeling and detection procedures were the same as those described for the Southern blot analysis. A Quant-iT™ PicoGreen® dsDNA reagent and kit (Invitrogen, Carlsbad, CA, USA) were used for the quantitation of h*GM-CSF* and *Dhfr*. The copy number ratio of h*GM-CSF* to *Dhfr* was estimated by comparison with the slots produced from a dilution series of standard h*GM-CSF* and *Dhfr* using Image J (National Institutes of Health).

Quantification of gene copy number of shotgun clone 1B20 and *ACTB* (β-actin) in CHO cell genome was performed by real-time PCR method. Genomic DNA was extracted from CHO DR1000L-4N and DG44 cells using DNeasy Blood &Tissue kit (Qiagen, Maryland, USA). Quantification was performed using the SYBR Green quantitative real-time PCR method with ABI PRISM 7700 (Applied Biosystems, Foster city, CA, USA) as described previously (4, 24). The primers used for real-time PCR were 5′-CACGAGTGACTCCCGAATGAA-3′, 5′-GTTTTCGGTACAACCATGAGGA-3′ (the amplified DNA size, 111 bp) and 5′-GCTGTGGGTGTAGGTACTAACAATGA-3′, 5′-GAATACACACTC-CAAGGCCACTTA-3′ (the amplified size, 80 bp) in shotgun clone 1B20 and *ACTB*, respectively. PCR reaction was performed with following thermal program: 10 min at 95 °C (1 cycle), 15 s at 95 °C and 1 min at 60 °C (40 cycles). Standard curves were constructed from known DNA solutions derived from shotgun clone 1B20 and *ACTB*.

Fluorescence *in situ* hybridization (FISH) Chromosome spreads were prepared from exponential-phase cultures using standard techniques. CHO cells were treated with colcemid and suspended in a hypotonic solution. Approximately 20 μl drops of the cell suspension were placed on a slide and dried with the metaphase spreader HANABI (ADSTEC, Funabashi-city, Chiba, Japan). Fluorescence *in situ* hybridization (FISH) was carried out as described previously (15, 22). CHO *ACTB* (β-actin), an insert of the shotgun clone 1B20 which is derived from previously constructed shotgun library of BAC clone Cg0031N14 (21), and the pSV2-dhfr/hGM-CSF vector were used as probes. *ACTB* and 1B20 were amplified by PCR with the primers 5′-ACACTTTCAGATCCAACTTTCAGTC-3′ and 5′-CAGAACTCAAAGGCAGTAAAGCTAC-3′, and 5′-TGTAAAACGACGGCCAGT-3′ and 5′-CAGGAAACAGCTATGACC-3′, respectively. The probes were detected using fluorescein isothiocyanate (FITC)-labeled streptavidin and/or an anti-DIG-rhodamine antibody. Chromosomes were counterstained with 4,6,-diamidino-2-phenylindole (DAPI) and observed under an Axioskop 2 fluorescence microscope (Carl Zeiss, St., Oberkochen, Germany). Photographs were taken with a CCD camera AxioCam MRm (Carl Zeiss).

RESULTS AND DISCUSSION

Screening from BAC library and analysis of specific chromosomal region adjacent to *Dhfr*-amplified region from CHO genome Previously, we constructed a CHO genomic BAC library from the CHO DR1000L-4N cell line and screened the BAC clone containing mouse *Dhfr* to determine the complete sequence of the DNA region containing exogenous *Dhfr* in the CHO DR1000L-4N genome (21). Nine clones (Cg0019N04, Cg0031N14, Cg0099A10, Cg0148O04, Cg0151K18, Cg0158C11, Cg0158M01, Cg0232F22, and Cg0338P23) were isolated by screening 22 HDR filters with radio-labeled mouse DHFR cDNA as a probe. We presumed that two clones (Cg0148O04 and Cg0338P23), which showed different *Hin*dIII fingerprinting patterns from 7 clones, contain CHO-derived endogenous *DHFR* and the other seven clones (Cg0019N04, Cg0031N14, Cg0099A10, Cg0151K18, Cg0158C11, Cg0158M01, and Cg0232F22) contain the CHO DR1000L-4N chromosomal region with exogenous mouse *Dhfr*. In this study, we also screened BAC clones containing the sequences of the integrated vector (pSV2-dhfr/hGM-CSF) from 22 HDR filters using DIG-labeled pSV2-dhfr/hGM-CSF as a probe. Fifteen positive signals (Cg0001H07, Cg0001B15, Cg0014B09, Cg0019N04, Cg0025E15, Cg0028O07, Cg0031N14, Cg0063E15, Cg0066E22, Cg0099A10, Cg0151K18, Cg0158C11, Cg0158M01, Cg0232F22, and Cg0291F21) were detected on the 22 HDR filters as candidate clones containing exogenous sequences derived from the integrated vector. Among 15 positive candidates, 7 clones (Cg0019N04, Cg0031N14, Cg0099A10, Cg0151K18, Cg0158C11, Cg0158M01, and Cg0232F22) were found to be the same as those isolated from the previous screening. Interestingly, neither of the two clones (Cg0148O04 and Cg0338P23) that might contain CHO endogenous *DHFR* was detected. In the mammalian system, amplicons range in size from a few hundred to 10,000 kilobases (kb) (16). Amplicons of various lengths and sequences in cell lines have been reported. Federspiel et al. (25) cloned an approximately 240 kb segment of DNA including and surrounding the amplified endogenous mouse *Dhfr* from a highly MTX-resistant murine cell line. Milbrandt et al. (26) estimated the total length of the unit repeat sequence to be 135 ± 15 kb pairs in the endogenous CHO *DHFR*-