Expression Vectors for Human-mouse Chimeric Antibodies

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The production of recombinant antibodies has been generally recognized as time-consuming and labor-intensive. The aim of our study is to construct mammalian expression vectors containing the cDNA encoding the human constant regions and murine variable regions to massively and cost-effectively produce full-length chimeric antibodies. Unique restriction sites flanking the Ig variable region were designed to allow for the replacement of variable regions generated by PCR. Western blot analysis of the chimeric antibodies revealed that the expressed products were of the predicted size, structure and specificity. The usefulness of the vectors was confirmed by construction of human-mouse chimeric antibody-HCAb which secretes murine antibody against the human colorectal cancer. Selected in medium containing gradually increasing methotrexate (MTX), clones with increased expression of the product gene can be efficiently generated. The secretion of recombinant chimeric antibody-HCAb yielded 30 pg cell−1 day−1 at 10−6 M MTX. With this high-level expression from pools, the convenient and rapid production of over 100 milligram amounts per liter of recombinant antibodies may be achieved, which indicates the significant roles of pYR-GCEVH and pYR-GCEVL in the production of chimeric antibodies.

Keywords: Antibody engineering, Chimeric antibody, Chinese hamster ovary cells, Eucaryotic expression, Expression vector

Introduction

The derivation of monoclonal antibodies using classic techniques has yielded many antibodies playing significant roles in both research and diagnosis (Persie et al., 1997). However, their inherent immunogenicity in patients had hindered their long-term administration in immunosuppressive therapy (Waldmann, 1991). Chimeric or humanized antibodies, which are largely human in composition, have been generated by recombinant DNA techniques (Liu et al., 1987; Burton, 1991; Harris et al., 1993). Recombinant antibodies have been increasingly used as therapeutics for a wide variety of diseases because their immunogenicity may be reduced by employing chimeric antibodies, in which the variable regions of murine antibodies are attached to human constant regions (Morrison, 1985). Thus, establishing high level expression system for chimeric antibodies is very significant.

For high level expression of recombinant antibodies, one of the most widely used mammalian expression systems is the gene amplification procedure offered by the use of dihydrofolate reductase-deficient Chinese hamster ovary cells (dhfr− CHO). The advantage of this system lies in its ability to amplify genes linked to DHFR, which leads to enhanced levels of protein expression. This system is based on DHFR gene coding for DHFR enzyme that catalyzes the conversion of dihydrofolate to tetrahydrofolate. MTX binds to DHFR enzyme, but dhfr− CHO cells, which take up an expression vector containing the DHFR gene, can develop resistance to it by amplifying the DHFR gene. Because the amplification unit is much larger (100 to 3000 kb) than the size of the DHFR gene, the specific gene of interest, which is cotinked to the DHFR gene in the same expression vector or adjacent resides in the host chromosome, can be coamplified (Kaufman et al., 1983).

Most vectors used for antibody expression in mammalian cells have evolved from the pSV2 series (Gorman et al., 1991). However, such vectors have not been optimized for chimeric antibody production. We present here new universal expression vectors that can be used to generate stable clones that consistently express high levels of secreted recombinant antibodies. We have tested the expression vectors using human-mouse chimeric antibody-HCAb, whose V (variable)
gene was obtained from hybridoma cells using RT-PCR. VH (variable region gene of the heavy chain) and VL (variable region gene of the light chain) were inserted into expression vector pYR-GCEVH and pYR-GCEVL, respectively. When subjected to successive rounds of selection in medium containing stepwise increments of MTX concentration, recombinant CHO cells with HCAb antibody productivity as high as 30 pg cell−1 day−1 were obtained. We found that the expression vectors can directly and highly express a variety of recombinant antibodies from pools of stable transfectants.

Materials and Methods

Construction of the heavy chain expression vector pYR-GCEVH The expression vector pYR-GCEVH was derived from the vector pSV2-dhfr (ATCC, USA) and pcdNA3 (Invitrogen, San Diego, USA). We chose pSV2-dhfr as framework, digested it with SphI endonuclease and isolated the larger fragment by gel electrophoresis. Thus, we deleted the 72 bp repetitive sequences of enhancer in SV40 promoter, and obtained the recombinant vector pYR-SV2-dhfr. Subsequently, the phCMV-MCS-BGH polyA fragment from pcdNA3 was excised and cloned into pYR-SV2-dhfr by BamHI and EcoRI to generate pYR-GSEVH. The expression vector pYR-GCEVH was made from pYR-GSEVH by insertion of human constant region (Cy or Cγ or Cδ or Cε) into Xhol site. Therefore, any variable region genes can be introduced into pYR-GCEVH by BamHI and NotI (Fig. 1).

Construction of the light chain expression vector pYR-GCEVL The Neo gene from pSV2-neo (ATCC, USA) was excised by HindIII and BamHI and cloned into pYR-SV2-dhfr to obtain pYR-SV2-neo, whose SV40 promoter was also weakened, for deletion of 72 bp repetitive sequences like pYR-SV2-dhfr. And then, excision of the phCMV-MCS-BGH polyA fragment and cloning into pYR-SV2-neo was performed to generate pYR-GSEVL. The plasmid pYR-GCEVL was obtained by insertion of human constant region (Cy or Cε) (Fig. 2).

Construction of expression vectors for Anti-human Colorectal cancer Antibody (HCAb) HCAb was recently developed in our laboratory (Yang et al., 2004), whose light and heavy chain variable region genes were obtained by RT-PCR, and cloned into plasmids by BamHI and NotI.

Cell culture, vectors transfection and selection COS-7 cells (ATCC, USA) were employed for transient antibody expression using LipofectAMINE™ (GibcoBRL, Life Technologies, USA) transfection reagent according to the manufacturer’s description. For stable transfection, dhfr− CHO (ATCC, USA) cells were chosen. Prior to transfection, dhfr− CHO cells were maintained in DMEM (Hyclone, Logan, USA) medium supplemented with 10% (V/V) FCS (GIBCO, Life Technologies, USA), L-glutamine (2 mM), glycine 10 µg ml−1, hypoxanthine 15 µg ml−1 and thymidine 5 µg ml−1 (GHT). Then, samples of 20 µg DNA of each expression vector were cotransfected into 107 dhfr− CHO cells using LipofectAMINE™ transfection reagent following the manufacturer’s instruction. After transfected, cells were cultured in nonselective medium for 3 days, then fed with GHT free DMEM medium that also contained 200 µg ml−1 of G418 (GIBCO BRL) to select for DHFR and Neo clones. After 3 weeks, selected clones were subcloned in 96-well microtiter plates. The culture supernatant was tested for the assembled antibody production by ELISA. And then, the transfected CHO clones were subjected to amplification (stepwise increments in MTX (Sigma, St. Louis, USA) level, such as 3 × 10−6, 10−6, 10−5 M) and subcloning at each level.

ELISA-assay ELISA analysis was used to assess the productivity of the secreted HCAb. The microtiter plate wells were coated overnight at 4°C with 10 µg L−1 of the goat anti-human kappa light chain (Bound and free) antibody (Sigma). Plates were blocked with 1% BSA in PBS for at least 2 h at 37°C followed by washing three times with PBS-0.1% Tween20 before culture supernatant was added overnight at 4°C. After extensive washing with PBS-Tween, goat anti-human IgG (Fc specific)-peroxidase antibody (Sigma) was added at the appropriate dilution and incubated for 2 h at 37°C. Further washes were followed by the addition of TMB (3,3',5,5'-tetramethylbenzidine) for 15 min at room temperature and 2 M sulphuric acid was used to stop the assay. OD450 was measured immediately.

Ig quantitation was examined by Elisa using purified human IgG as a standard. The supernatant harvested from 107 cells, cultured for 24 h, was assayed to determine the level of production of chimeric antibody HCAb by ELISA (see above). The amount of the antibody was assessed by comparison to a standard curve.

Western blot analysis of recombinant Ig molecules Standard procedures were used to analyse the weight of the recombinant Ig molecules. IgG HCAb from 20 ml of culture supernatants was purified using a protein-A column (Pierce, Rockford, USA), concentrated using Centricon filtration (Millipore, Bedford, MA) and then subjected to SDS-PAGE under reducing conditions (Laemmli, 1970). Finally, the molecular weight of the individual Ig chains was estimated by western blot using specific peroxidase conjugated anti-human gamma or kappa antibody.

Result

Construction of Ig light chain and heavy chain expression vectors Two vectors, pYR-GCEVH for Ig heavy chain and pYR-GCEVL for Ig light chain, were constructed (Fig. 1, 2). The vectors contain BamHI and NotI endonuclease restriction sites flanking the rearranged V region cDNA generated by PCR. These two vectors contain the hCMV promoter, BGH polyadenylation site, C region, and origin of replication. As pYR-GCEVH contains the weakened DHFR gene, while pYR-GCEVL contains the Neo gene, co-transfection of the two plasmids into mammalian cells warrants the production of functional, complete Ig molecules of desired antigen binding specificity.

Chimeric antibody production by transfected cells The dhfr− CHO cells were transfected with pYR-GCEVH and

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pYR-GCEVL by LipofectAMINE. Stable transfectants were selected by culturing the cells in GHT free DMEM medium with 200 µg ml⁻¹ of G418. Secretion of the chimeric antibody HCAb was assayed by ELISA. The production was over 1 µg ml⁻¹ before amplification.

**Methotrexate amplification** Significant improvement of expression level in CHO cells was achieved with vectors amplification. The amplified region is usually larger than that occupied by the marker gene, which resulted in the coamplification of associated sequences including the gene encoding the product (Liu et al., 2004). The target gene was cloned into the multiple cloning sites with transcription initiated by the human cytomegalovirus promoter sequence, while transcription of the DHFR gene was triggered by a weakened SV40 promoter. In order to highly express recombinant antibodies in CHO cells, the specific DHFR inhibitor MTX was added to pools of transfected cells at concentrations between 3 × 10⁻⁸ and 10⁻⁶ M. We found that the DHFR-selection system usually produced relatively high-level expression of chimeric antibodies after only a single round of gene amplification. The average productivity of selected clones at 3 × 10⁻⁸ M MTX was over 30 µg ml⁻¹, with the highest production of 100 µg ml⁻¹ of HCAb at 10⁻⁶ M MTX.

**Western blot analysis of recombinant Ig molecules** Two
additional analyses were performed to ensure the correct structure and size of the recombinant antibodies. Firstly, an ELISA indicates the secretion of the fully assembled H chain and L chain from transfected cells. Secondly, western blot was performed to determine the molecular weight of recombinant Ig chain (Fig. 3). Fig. 3 shows that the the size of Ig H chains are approx. 50 kDa as expected, with the size of Ig L chains approx. 21 kDa. These results, together with the ELISA data, confirmed the correct structure of the individual Ig chains, thus demonstrated the correct polypeptide assembly of the heavy and light chains in the production of the recombinant Ig molecules.

**Discussion**

The production of Ig molecules with desired specificities has become an important area of medical and basic research (Mclean *et al.*, 2000). Currently, the process of generating cell lines that produce high levels of recombinant antibody...
amplified 500 to 2000 folds in dhfr clones. It is generally accepted that DHFR gene can be minimim ize the amount of DHFR to allow selection of stable maximize the production of recombinant product and to immmunoglobulin isotypes.

express the variable regions of antibodies in different can both transiently and stably express antibodies; (c) they can chim eric antibodies by exchanging their V regions; (b) they characterize: (a) they can easily express any hum an-m ouse

Furtherm ore, with careful designing and construction, the was adopted because the heavy and light chain coding system s have been frequently utilized, one of which is a two- vector system with both Ig chains on the same plasmid (Page et al., 1994), the other being the one-vector system (Page et al., 1991; Newman et al., 1992; Tada et al., 1994). In our study, the two-vector system was adopted because the heavy and light chain coding sequences could be more easily inserted into the two vectors. Furthermore, with careful designing and construction, the vectors used in this study had already possessed the following characteristics: (a) they can easily express any human-mouse chimeric antibodies by exchanging their V regions; (b) they can both transiently and stably express antibodies; (c) they can express the variable regions of antibodies in different immunoglobulin isotypes.

The expression vectors in this study have been optimized to maximize the production of recombinant product and to minimize the amount of DHFR to allow selection of stable clones. It is generally accepted that DHFR gene can be amplified 500 to 2000 folds in dhfr CHO cells, which consequently leads to the 500- to 2000- fold amplification of the colinked target gene, and finally the several-hundred-fold amplification of the productivity of recombinant protein. Since the target genes are always present at the telomeric ends of amplified arrays in the chromosomes after long-term culture in the absence of MTX, the transfected cells have better hereditary stability (Kim et al., 1999). This mechanism inspired us to use the weakened DHFR gene amplification procedure to increase the recombinant proteins production in lower MTX level.

We chose initial transcription of the immunoglobulin gene from hCMV promoter to facilitate strong expression in mammalian cells, with the optimized peptide, NH2-Met-Tyr-Phe-Ser-Ala-Ile-Val-Ser-Ala-Ser-Leu-Ser-COOH, as a signal. The incorporated SV40 ori enabled the high episomal replication in COS cells and hence the transient expression. Most vectors are generally believed to achieve either transient expression or stable expression. However, the vectors in our study, pYR-GCEVH and pYR-GCEVL, were found not only to express antibodies transiently within 2 days after transfection, permitting preliminary testing of the product, but also to stably express the products for the long term extensive testing. Furthermore, the vectors were discovered to be able to express different isotypes of Ig because they contain restriction sites facilitating the exchange of the C regions. Any antibody isotype may be combined with the V region chosen by constructing hum an-m ouse chim eric antibody-HCAb (recently found in our laboratory). After 3 rounds of screening, the heavy and light-chain expression vectors respectively, may balance expression of two chains of antibodies. Second, selecting with two markers may help to delete the pseudo-clones. However, due to the lack of detailed knowledge in the events leading to the integration of nonhomologous plasmids into chromosomal DNA of CHO cells (Kim, 1998), the disproportionate amplification and/or expression of Ig chains may be a drawback in using a two-vector system (Trill, 1995) for it is difficult to control the proportional amplification and/or expression of each Ig chain. In order to overcome this drawback, we attempted to express more heavy chains by including the weakened DHFR gene in the heavy chain expression vector to balance the expression of the heavy and light chains because most IgG-producing mouse myeloma tumors and cell lines are known to synthesize more light chains than heavy chains. Furthermore, the cells with proportional amplification and/or expression of Ig chains can be obtained by extensive screening. To achieve this aim, the constructed expression vectors in our study were cotransfected into DHFR-deficient CHO cells. After initial selection, the parental clone was subjected to DHFR-mediated gene amplification in medium containing stepwise increments in MTX followed by limiting dilution cloning to identify the highest producer subclones at each MTX level.

In this study, the antibody output of the most productive subclone producer was found to be rapidly increased when MTX level rose. We tested the usefulness of the vectors by constructing human-mouse chimeric antibody-HCAb (recently found in our laboratory). After 3 rounds of screening, the secretion of recombinant chimeric antibody-HCAb was found to reach over 100 mg L−1 (30 pg cell−1 day−1) at 10−7 M MTX. The Ig chains were also demonstrated to retain correct H and

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**Fig. 3.** Western blot analysis of purified chimeric antibody HCAb. Recombinant IgG (20 μg/lane) purified from culture supernatants of the transfected cells were subjected to SDS-PAGE analysis under reducing conditions. Lane 1, the band of human γ chain (50 kDa). Lane 2, the band of human κ chain (21 kDa).
L chain assembly by western blot analysis. These two findings indicated that the vectors highly expressing chimeric monoclonal antibodies had been successfully constructed, which would consequently facilitate the production of functional recombinant Ig with exchanged V regions.

In conclusion, we present here a study of the mammalian expression system that is less time-consuming and more cost-effective in the mass production of full-length antibody molecules in stable pools. Unlike most previously described antibody expression systems, our vectors have been proved to have the following characteristics: (a) they can easily express any human-mouse chimeric antibodies by exchanging their V regions; (b) they can both transiently and stably express antibodies in eukaryotic cells; (c) they can express the variable regions of antibodies in different immunoglobulin isotypes; (d) they can balance the expression of two chains of antibodies; and (e) they can enable the effective amplification and the mass production of recombinant antibodies, providing a possible solution to the bottlenecked recombinant antibody expression.

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References


