Production of recombinant granulocyte colony-stimulating factor by knocking into the active immunoglobulin heavy chain gene locus in the hybridoma cell line

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Abstract

The hybridoma cell line KM50 originally produces a monoclonal antibody at a concentration of $\sim 40 \text{ mg ml}^{-1}$ in ascites. To investigate the possibility to apply this expression system to the production of useful proteins, the cDNA encoding human granulocyte colony-stimulating factor was inserted by homologous recombination into just downstream of the promoter of the active immunoglobulin heavy chain gene of KM50. Site directed integration of targeting DNAs resulted in the disruption of expression of the immunoglobulin heavy chain proteins with a frequency of 1 in $10 \sim 100$ G418-resistance transfectants. One of the monoclonal antibody-deficient transfectants produced 25 ng ml^{-1} of granulocyte colony-stimulating factor in the supernatant of its cell culture the number of molecules of which corresponds to that of the monoclonal antibody originally produced by KM50. However, when this transfectant was injected intraperitoneally, it produced only a $9 \mu\text{g ml}^{-1}$ concentration of granulocyte colony-stimulating factor in ascites, which is approximately 3 orders of magnitude less than the monoclonal antibody. This method may be applicable to production of other recombinant proteins, although further optimization in the conditions of production would be needed in order to reach much higher yields.

Abbreviations: G-CSF – granulocyte colony-stimulating factor, IgH – immunoglobulin heavy chain, G418^R – G418-resistance, VH – immunoglobulin heavy chain variable region, μE – IgH intron enhancer, HSA – human serum albumin, NEO – neomycin resistance/G418^R, Atk – herpes simplex virus thymidine kinase gene

Introduction

One of the challenges created by modern biotechnology is to open the ways to mass production of useful proteins which are naturally expressed at a very low level. Immunoglobulin production in myeloma or hybridoma cells is very efficient, suggesting that the immunoglobulin gene locus on these cells may be adapted to an efficient system for recombinant protein production. Indeed, immunoglobulin expression system is currently being tested for the production of useful recombinant pharmaceuticals such as human

growth hormone and immunoglobulin Fv molecules (Mitchell et al. 1991; Xiang 1992).

Transcription of the immunoglobulin heavy chain (IgH) gene is mainly regulated by elements within the immunoglobulin heavy chain variable region (VH) promoter and the IgH intron enhancer (μE) (Staudt and Lenardo 1991). Many eukaryotic expression vectors use these control elements, although the level of produced recombinant proteins is often lower than that of original IgH proteins which are produced in hybridoma cell lines, indicating that there are probably additional elements involved in the regulation of

IgH gene expression levels (Mocikat et al. 1995; Furebring et al. 1997; Oancea et al. 1997; Chauveau et al. 1998). Furthermore, the expression of foreign genes in transfected cells is generally unpredictable as transgenes are integrated randomly into the host cell genome. In many cases, transgene expression is suppressed by neighboring chromatin structures or by the repeated nature of the multiple transgene copies present at the integration site (Kolb et al. 1999). Therefore, we have sought to specifically target a foreign gene into the IgH locus by homologous recombination.

Homologous recombination is an efficient way to introduce foreign genes into a desirable site on the host cell genome. This recombination strategy will automatically provide the inserted gene with the appropriate chromatin structure and regulatory elements required for its abundant expression and overcomes many of the difficulties associated with randomly integrated transgenes. To date, homologous recombination in hybridoma cells has been successfully used to direct the production of fusion proteins consisting of a monoclonal antibody with mouse-human chimeric antibody chains (Fell et al. 1989; Kardinal et al. 1995; Mocikat et al. 1997). For example, the murine hybridoma cell line L20 was targeted at the active immunoglobulin heavy chain constant region (CH) locus with a vector containing human C γ 1 gene and the transformed clone produced almost the same amount of mouse-human chimeric antibody as its original murine antibody in cell culture (Fell et al. 1989). To our knowledge, however, there has been no study investigating whether heterologous proteins other than antibodies could be produced in the similar amounts to original monoclonal antibodies in hybridoma cells.

KM50 is a hybridoma cell line which produces a rat anti-human serum albumin (HSA) monoclonal antibody (IgG1) (Yoshida et al. unpublished results). KM50 yields a large amount of this monoclonal antibody, in optimal conditions reaching 40 mg ml⁻¹ in ascites fluid. Therefore, this prompts us to study whether this expression system may be applicable to efficient production of heterologous proteins.

In the present study, we tried to insert human granulocyte colony-stimulating factor (G-CSF) cDNA into the active IgH locus of KM50 as a model case, because G-CSF is a practically useful cytokine which has been successfully used to treat neutropenic patients in a variety of clinical situations (Nagata et al. 1986; Komatsu et al. 1987; Kuga et al. 1989; Moore

1991). Theoretically, this approach can be applied to other genes of interest, e.g. those encoding pharmaceutically relevant proteins.

Materials and methods

Cloning of active VH-gene containing fragment

Cloning, subcloning and sequencing of DNA were carried out using standard protocols (Sambrook et al. 1989). Genomic DNA was isolated from the hybridoma cell line KM50 that produces the rat anti-HSA monoclonal antibody. The active rat VH gene-containing fragment (9.3 kb: containing both the promoter and μ E) of KM50 was identified as a rearranged band in Southern hybridization of *Xba*I-digested DNAs with mouse JH probe (Maki et al. 1980; Brüggemann et al. 1986). A DNA fragment purified from agarose gel was cloned into the *Xba*I site of λ ZAP (Stratagene, La Jolla, CA). The phage library of these genomic DNAs was screened with the mouse JH probe. The DNA sequences of the promoter and the coding regions of the active VH of KM50 are described in Figure 1.

Construction of targeting vectors

The structure of the targeting vectors is described in Figure 2. Briefly, to produce a unique *Sal*I site at just the downstream region of the active VH promoter, the cloned rat VH gene was first deleted with BAL31 nuclease from the *Nco*I site at the initiation codon ATG and ligated with a *Sal*I linker. This *Sal*I site is used for insertion of heterologous genes. The *Hind*III site in the first intron between exon 1 (leader exon: L exon) and exon 2 (VDJ) of the VH gene was converted to the *Cla*I site with a linker. The vector pHoKM17 allows the expression of neomycin resistance/G418^R (*NEO*) gene under the control of KM50 active IgH promoter. A 1.7 kb fragment containing both the *NEO* gene and the polyadenylation signal of the herpes simplex virus thymidine kinase gene (Atk), derived from pAGE103 (Mizukami and Itoh 1987), was inserted into the *Sal*I-*Cla*I to replace the L exon of the active VH gene of KM50. To increase the frequency of G418^R transfectants, the sequence flanking the translation initiation codon ATG of the *NEO* gene was changed to the best context for the initiation reported by Kozak (1986, 1991) with a synthetic *Sal*I site- and ATG codon-

Figure 1. DNA and amino acid sequences of the KM50 active VH region. Capital letters represent the coding region and small letters represent the non-coding region.

containing oligonucleotide 5'-GTCGACCATGG-3'. A fragment (1.1 kb) containing μ E is deleted by digestion with *EcoRV*. The vector pHoKM18 is similar to pHoKM17 except for containing the μ E region. The vector pHoKM23LRCSF21 was constructed as follows. To install a drug selection marker, a 2 kb *EcoRV*-*Bam*HI fragment of pAGE28 (Mizukami and Itoh 1987) (containing the tandem promoter sequences consisting of pBR322 P1 promoter and the herpes simplex virus thymidine kinase gene promoter, *NEO* gene and *Atk*) was inserted into the *Bgl*III site (located in the upstream region of KM50 active VH promoter) using a *Bgl*III linker. The first *Pst*I site from the initiation codon ATG at the 5' non-coding region and *Dra*I site at the 3' non-coding region of the G-CSF cDNA (0.7 kb) (Nagata et al. 1986; Komatsu et al. 1987) were converted to *Sal*I sites using *Sal*I linkers. A DNA fragment encoding the *Atk* was derived from a 0.6 kb pHSG274 *Sma*I-*Hind*III fragment (Brady et al. 1984). Both the *Sma*I and the *Hind*III sites were changed to *Sal*I and *Cla*I sites with the linkers, respectively. Both the 0.7 kb *Sal*I fragment which encodes the whole coding region of G-CSF and the 0.6 kb *Sal*I-*Cla*I fragment containing the

Atk were inserted into the *Sal*I and the *Cla*I sites to replace the L exon of the active VH gene of KM50. The vector pHoKM24LRCSF21 is similar to pHoKM23LRCSF21 except containing the μ E.

Transfection of targeting DNAs

The DNA of either targeting vector was linearized by *Xba*I and *EcoRV* digestion. The DNA fragment containing the IgH promoter and heterologous genes was isolated from agarose gel and used for transfection experiments described below. Two μ g of the DNA fragment and 2×10^6 KM50 cells were used for each transfection. Transfection was carried out by electroporation using a Gene Pulser apparatus (Bio-Rad laboratories, Richmond, CA) at 200 V and 250 μ F. After the transfection, cells were plated into 96-well tissue culture plates at a density of 10^4 cells/well. The selection agent G418 (Gibco BRL, Gaithersburg, MD) was added 48 hr later at a final concentration of 0.5 mg ml⁻¹. G418^R transfectants were obtained and recloned. After the wells became confluent with the recloned cells, supernatants were assayed for the expression of the anti-HSA monoclonal antibody and

Figure 2. Structures of targeting vectors. Vectors were constructed as described in Materials and Methods. Briefly, *NEO*-*Atk* gene replaced the L exon of the active VH gene of KM50 in pHoKM17 and pHoKM18. In pHoKM23LRCSF21 and pHoKM24LRCSF21, the G-CSF-*Atk* gene replaced the L exon of the active VH gene and the P-*NEO*-*Atk* gene was inserted into the *Bgl*II site in the upstream region of the VH promoter as a transformation selection marker. NEO, Tn5-derived G418 resistance gene; *Atk*, transcription termination / polyadenylation signals from herpes simplex virus thymidine kinase gene; IgP, active immunoglobulin heavy chain promoter; L, immunoglobulin heavy chain leader exon; VDJ, immunoglobulin heavy chain variable region exon; μ E, immunoglobulin heavy chain enhancer; P, the tandem promoters of herpes simplex virus thymidine kinase gene and P1; CSF, G-CSF cDNA. Restriction enzyme sites are described partially.

G-CSF. Monocloned cells were propagated as ascites in Pristane (Aidrich Chemical Co., Inc., Milwaukee, U.S.A.)-treated nude mice (BALB/c nu/nu). The experimental protocols to use animals were approved by the Animal Ethics Committee at Kyowa Hakko Kogyo, Co., Ltd.

Quantification of anti-HSA monoclonal antibody KM50

96-well microtiter plates were coated overnight with $10 \mu\text{g ml}^{-1}$ HSA (SIGMA, Missouri, U.S.A.) at 4°C . After removal of the HSA solution, $50 \mu\text{l}$ of growth medium samples from transfectants or diluted-ascites fluids of the nude mice containing the anti-HSA monoclonal antibody KM50 at various concentrations were added to the wells and incubated overnight at 4°C . After these were washed with Tween-PBS, $50 \mu\text{l}$ ($10 \mu\text{g ml}^{-1}$) of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO Japan Co., Ltd., Kyoto, Japan) was added to each well and then washed four times with Tween-PBS. 3,3'-diaminobenzidine tetrachloride/nickel-cobalt kit (ZYMED Laboratories, INC. CA, U.S.A.) was used for the detection. For quantification of the anti-HSA monoclonal antibody, the purified monoclonal antibody KM50 was used as a standard. Total protein

quantification was carried out using the bio-rad DC protein assay kit (Bio-Rad laboratories).

Quantification of G-CSF

For sandwich ELISA, 96-well EIA plates (Flow laboratories, Inc., McLean, U.S.A.) were coated with $50 \mu\text{l}$ of rabbit anti-human G-CSF polyclonal antibody (produced in Kyowa Hakko Kogyo Co. Ltd.) ($10 \mu\text{g ml}^{-1}$) at 4°C for 2 hr. After removal of the antibody solution, residual protein-binding sites on the plates were blocked with $100 \mu\text{l}$ of 1% BSA-PBS. Fifty μl of growth medium samples from transfectants or diluted-ascites fluids of the nude mice containing G-CSF at various concentrations were then added to the wells and incubated overnight at 4°C . After these were washed with Tween-PBS, $50 \mu\text{l}$ ($10 \mu\text{g ml}^{-1}$) of the mouse anti-human G-CSF monoclonal antibody KM341 (Yoshida and Shitara 1989) was added to each well as the second antibody and incubated overnight at 4°C . Horseradish peroxidase-labeled rabbit anti-mouse immunoglobulins (DAKO Japan Co., Ltd.) were used as the third antibody. Plates were then washed four times with Tween-PBS. 3,3'-diaminobenzidine tetrachloride/nickel-cobalt kit (ZYMED Laboratories, INC.) was used to produce a color change. For quantification of G-CSF, purified

recombinant G-CSF (Kuga et al. 1989) was used as a standard.

SDS-PAGE and Western blot analyses

Growth medium of transfectants and ascites was analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition. The protein bands were visualized with Coomassie Blue R-250. Western blotting for the detection of KM50 immunoglobulins or G-CSF was performed as described previously (Yoshida and Shitara 1989). Briefly, growth medium samples (20 μ l) or ascites samples (2 μ l each) were separated by SDS-PAGE, and transferred to nitrocellulose membranes. For the detection of the immunoglobulins, membranes were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins. For the detection of G-CSF, the membranes were incubated with the monoclonal antibody KM341 (10 μ g ml⁻¹), followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG (DAKO Japan Co., Ltd.). The immunoreactive bands were visualized with a 3,3'-diaminobenzidine tetrachloride/nickel-cobalt kit (ZYMED Laboratories, INC.).

Results and discussion

Structures of KM50 active VH genomic DNA

The genomic library of KM50 was generated and the active VH gene was cloned. The nucleotide sequence of the VH was determined. As shown in Figure 1, the cloned 9.3 kb-*Xba*I fragment contains the whole coding sequence of the VH and μ E. The active VH gene of KM50 is composed of VDJ2 recombination with the V segment belonging to rat VH-1B family (Brüggemann et al. 1986; Kabat et al. 1987). There is a octamer sequence, TATA box and cap site sequences at the 5' non-coding region of the cloned VH gene, indicating that this clone contains the promoter region.

Analysis of recombination events in KM50 cells

To assess the frequency of homologous recombination in KM50, we constructed the targeting vectors pHoKM17 and pHoKM18 (Figure 2). In these vectors, the *NEO* gene replaces the L exon of the VH

gene, so that the expression of the *NEO* gene is under the control of the VH gene promoter generates G418^R transfectants. We used the enhancer trap method (Jasin and Berg 1988) to enrich homologous recombinants by reducing the frequency of transfectants that have incorporated the donor DNA fragment at other sites. The vector pHoKM17 is enhancerless, so that fewer random insertions can express sufficient amount of the *NEO* gene at a sufficiently high level to confer resistance to G418. If the targeted locus provides the missing enhancer activity, the fraction of G418^R transfectants that were targeted would be greater than for the enhancer positive vector pHoKM18. Both vectors have 6.1 kb of homology on the 5' side, whereas 2.1 kb and 3.1 kb of homologies on the 3' side of the inserted *NEO*-Atk gene are present in pHoKM17 and pHoKM18, respectively.

We have tested the efficiency of this technique for enriching targeted recombination in the VH locus. When we used the donor DNA fragment derived from pHoKM18, 190 G418^R colonies were obtained. On the other hand, only 90 G418^R colonies were obtained even when fourfold the number of cells were treated with the donor DNA fragment of pHoKM17 under similar conditions (Table 1). These results demonstrate that the enhancerless *NEO* gene construct reduces the number of G418^R transfectants by ~10-fold compared to that obtained with the enhancer-bearing *NEO* gene construct. This reduction supports our expectation that the enhancerless gene will only be expressed at a restricted number of loci.

Site directed integration into the active IgH locus of KM50 should be expected to result in deficiency of the anti-HSA monoclonal antibody production. To test whether the vectors had integrated into the IgH locus, we assayed by ELISA the culture medium of 69 G418^R transfectants which were transfected with the donor DNA fragment of enhancerless vector pHoKM17. Figure 3 shows, 7 of 69 transfectants produced no anti-HSA monoclonal antibody. Furthermore, although IgH production in these seven transfectants was abolished, the IgL production remained normal both in the medium (Figure 4) and in the cell bodies by Western blotting analysis. This suggested that the IgH gene has been knocked out in these cells.

These experiments suggest that the hybridoma cell line KM50 is capable of mediating homologous recombination events at high frequencies and that this feature can be used to modify the IgH locus within the genome of KM50 to direct production of heterologous proteins.

Table 1. Frequency of targeted disruption of the active IgH gene in KM50

Plasmid	Treated cells	G418 ^R colonies		$\mu\text{E}(-) / \mu\text{E}(+)$ Frequency ratio	MoAb(-) clones	
Number	Frequency*	Number	Frequency**			
pHoKM17	6.4×10^7	90	2.8	-	7***	0.10
pHoKM18	1.6×10^7	190	24	0.12		0.012****

Linearized donor DNAs were transferred by electroporation to KM50 cells, which were then incubated in normal medium. At 2 to 3 days after electroporation, cells were plated in microtiter wells at limiting dilution in selective medium (500 μg of G418 per ml) to measure the frequency of G418^R transfectants. These selected colonies were then assayed for the production of anti-HSA antibody. Numbers are the sum of 5 independent experiments. * Number of G418^R transfectants in 2×10^6 treated cells. ** Number of anti-HSA monoclonal antibody negative clones in G418^R transfectants. *** 69 colonies of 90 G418^R transfectants were assayed for the production of anti-HSA monoclonal antibody by ELISA (Figure 3). **** Expected number calculated by $\mu\text{E}(-) / \mu\text{E}(+)$ frequency ratio.

Expression of recombinant G-CSF in cell culture

Hybridoma cell line KM50 was then tested for the production of G-CSF by transfecting human G-CSF cDNA under the control of KM50 active VH promoter (Figure 2). The P (tandem promoter of herpes simplex virus thymidine kinase gene and P1)-*NEO*-Atk gene which served as the selection marker was inserted into the *Bgl*/III site located in the upstream region of the active VH promoter of KM50. Although we con-

structed this type of vector by inserting P-*NEO*-Atk gene in both directions, we could not efficiently obtain transfectants by the vector which contains the *NEO*-Atk gene and G-CSF-Atk gene in the same orientation.

We obtained G418^R transfectants with the DNA fragment of μE containing vector pHoKM24 LRCSF21. One of those transfectants, #6801, produced both anti-HSA monoclonal antibodies (almost the same amount as that produced by KM50) and

Figure 3. Secretion of anti-HSA monoclonal antibody from KM50 transfectants. Culture medium of each transfectant was subjected to ELISA analysis as described in Materials and Methods. A1–A12, purified anti-HSA monoclonal antibody was doubly titrated from 10 $\mu\text{g ml}^{-1}$ at A12 to A1 (positive control); B1–B11 (11 clones), culture medium of G418^R transfectants with the DNA fragment of pHoKM18; B12–H8 (69 clones), culture medium of G418^R transfectants with the DNA fragment of pHoKM17; H9, H10, culture medium of non-transfected KM50 cells; H11, H12, blank.

Figure 4. Western blot analysis of the rat monoclonal antibody produced by KM50 or its transfectants. Growth medium of three independent isolated clones were separated by SDS-PAGE and blotted onto nitrocellulose filter as described in Materials and Methods. M, molecular weight markers of 92.5, 66, 45, 31, 21.5 and 14.4 kd.

G-CSF (8.5 ng ml^{-1} in culture supernatant), suggesting that the donor DNA was not targeted to the IgH locus. Using the enhancerless vector pHoKM23LRCSF21, we obtained the anti-HSA antibody non-producing (HSA(-)) transfectants with the frequency of 5 clones in 656 G418^R transfectants.

The monocloned cells were grown to high density ($\sim 10^6 \text{ cells ml}^{-1}$) and their culture supernatants were then analyzed by ELISA and Western blotting to assess the production of anti-HSA monoclonal antibody and G-CSF. Further analyses revealed that two of the transfectants (#6801, #69155), that were scored as G-CSF positive in the ELISA, produced it with the electrophoretic mobility expected for full length G-CSF in ascites (Figure 5).

From ELISA analysis we estimate that the culture supernatant of the maximum producer clone #69155 contained $\sim 25 \text{ ng ml}^{-1}$ while the low producing transfectant #69159 yielded $\sim 1.2 \text{ ng ml}^{-1}$ (data not shown). Because the molecular weight of immunoglobulin (150 kd) is one order of magnitude higher than that of G-CSF (19 kd), we estimated that the actual amount of G-CSF molecules produced by

Figure 5. Western blot analysis of human G-CSF produced in ascites fluids by KM50 transfectants. M, molecular weight markers of 92.5, 66, 45, 31, 21.5 and 14.4 kd. rG-CSF: purified recombinant human G-CSF. A few upper bands of G-CSF band are likely to represent cross-reactants of immunoglobulins produced by nude mice because peroxidase-conjugated rabbit anti-mouse IgG was used as the second antibody.

#69155 was equivalent to that of the anti-HSA antibody by KM50 cell line.

Expression of recombinant G-CSF in ascites

Four transfectants (KM50, #6801, #69155, #69159) were then intraperitoneally injected to assess the amount of G-CSF proteins which those transfectants yield in ascites (Table 2). Ascites developed by KM50 and its transfectants contained almost same amount of total proteins ranging from $29\text{--}34 \text{ mg ml}^{-1}$ at the concentration. These results suggest that both clones expanded to the same degree in the abdominal cavity. Among these clones, clone #6801 yielded $\sim 10 \text{ mg ml}^{-1}$ at concentration which is comparative to KM50. However, clone #69155 and #69159 produced no anti-HSA monoclonal antibodies in ascites representing the character of these transformed cells in *in vitro* culture.

Surprisingly, both clones of #69155 and #69159 only produced at the maximum of $\sim 9 \text{ } \mu\text{g ml}^{-1}$ G-CSF. This is three orders of magnitude lower than the level of anti-HSA monoclonal antibody produced by KM50. One possible reason for this inefficient production *in vivo* is that homologous recombination between transferred and chromosome DNA might be incomplete. In this context, however, it is unlikely that any such general incompleteness would cause drastic changes in gene expression, because almost a

Table 2. Recombinant G-CSF production in the ascites of nude mice implanted with KM50 transfectants.

Animal name	Ascites volume (ml)	Total protein (mg ml ⁻¹)	anti-HSA MoAb (mg ml ⁻¹)	G-CSF (μ g ml ⁻¹)
KM50 (16)	0.85	28	5	0.2 >
KM50 (19)	2.0	29	10.5	0.2 >
6801 (16)	0.1(†)	33	11	5.2
69155-1 (16)	0.8	27	0.1 >	6.0
(19)	1.8	30	0.1 >	8.5
69155-2 (16)	1.4	33	0.1 >	5.7
(19)	2.5	39	0.1 >	9.0
69159-1 (27)	2.0	33	0.1 >	0.2 >
(30)	2.0	29	0.1 >	0.2 >
69159-2 (27)	0.8	24	0.1 >	1.4

(†): died when ascites was collected. Each mouse received a single intraperitoneal injection of 1×10^6 cells in a total volume of 0.5 ml. Animals were tapped, depending on the clinical appearance and rate of ascites production of individual mice, with a maximum of two taps per mouse. The numbers in parentheses indicate the day after the implantation, when the ascites was collected.

comparable number of G-CSF protein molecules to that of original KM50 monoclonal antibody was calculated to express *in vitro*. Another possible reason is that the presence of the *NEO* gene in the upstream of the VH promoter might interfere with promoter activity. In the previous studies by other groups (Sun et al. 1994; Oancea et al. 1997; Mocikat 1999), although the known regulatory elements remained unaffected in IgH loci, the original immunoglobulin production rate was maintained only in a fraction of the recombinant transfectants after the introduction of the selection marker gene. Moreover, this variation of production rate was recovered by removal of the marker gene. These results suggested that the promoters of the marker gene and the IgH gene compete for some limiting elements and that removal of the marker gene from the chromosome would increase IgH expression to the normal level. Therefore, it is possible that the same mechanism might play a similar role in our case and then the solution might be to eliminate the selection marker *NEO* gene. Another possible reason is that G-CSF might be unstable in ascites. G-CSF is a glycoprotein produced by a variety of cell types, including endothelial cells and macrophages. It is mainly secreted into the plasma and presumably not so stable in ascites fluid that is rich in a number of types of proteases (Cruz et al. 1998). This suggests that the system might be more suitable for the production of recombinant proteins (e.g. IL-10, IL-12, antibodies) that are stable in ascites (Zeimet et al. 1998).

In conclusion, we have carried out site directed integration to produce G-CSF under the control of an IgH promoter. The number of the molecules is comparative to the original antibody in cell culture but much lower in ascites. This method may be applicable

to the production of other recombinant proteins, although further optimization of the conditions of production is needed in order to reach much higher yield in ascites.

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