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(54) Title: EXPRESSION SYSTEM FOR EUKARYOTIC CELL LINES

(57) Abstract

It is disclosed a process for the preparation of recombinant human G-CSF for which is used a vector system which comprises at least one of the following elements: (1) a CMV promoter upstream of a DNA sequence encoding the desired protein, (2) a sequence for processing of RNA transcripts, and (3) a selection and amplification marker sequence; it is furtherly disclosed the use of such recombinant human G-CSF for the preparation of pharmaceutical compositions for promoting hematopoiesis, for the augmentation of the defence mechanism against infection and for the stimulation and hyperproduction of functionally primed effector cells against malignant diseases.

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EXPRESSION SYSTEM FOR EUKARYOTIC CELL LINES

Field of the invention

The invention relates to a process for the production of recombinant proteins in eukaryotic cell lines. In particular, it relates to the application of an expression vector system to the production of stable eukaryotic cell lines, which are able to synthesize large quantities of active proteins, correctly processed in the post-translation phase and having a relevant therapeutic value.

State of the art

Techniques for directing the synthesis of therapeutically important eukaryotic proteins in bacteria and yeasts are well established. Such methods are efficient but involve problems due to incorrect post-translational modifications (protein processing, glycosylation, myristilation, gamma-glutamyl-carboxylation, etc.). In addition, many proteins are incorrectly folded and may form insoluble aggregates.

These problems can be solved by using eukaryotic cell lines [COS cells, Chinese hamster ovary (CHO) cells , NIH-3T3 cells] as hosts for the production of recombinant proteins. Vectors useful for the transfer and expression of heterologous genes in eukaryotic cells usually carry viral promoters and enhancers in order to direct transcription. In addition, the cDNA sequence that one intends to express is usually equipped with a polyadenylation signal, and in some vectors, with an intron, because splicing (the process of removing introns and joining of exons) seems to be essential, in some cases, for the DNA expression. Polyadenilation signal and intron sequences are the sequences involved in the processing of transcripts. In addition to the aforesaid, eukaryotic vectors usually contain a prokaryotic replication origin and markers which enable

selection both in eukaryotic and prokaryotic cells.

A problem facing many eukaryotic expression systems is the low yield of recombinant protein if compared to that of prokaryotic systems. A large number of variables may contribute to poor production of the recombinant protein: poor vector stability; low vector copy number; inefficient promoters or enhancers; strong promoter/enhancer combinations that may interfere with vector replication and cause instability; lack of polyadenylation sites; lack of RNA splice sites; inefficient transport of mRNA from nucleus to cytoplasm; undesirable mRNA secondary structures; unsuitable selective markers resulting in low selective pressure for plasmid maintenance; mRNA instability; protein degradation; incorrect protein processing or folding. The interaction of these factors is yet poorly understood and the vectors construction designed for optimal yield of recombinant protein in eukaryotic cells, have yet to be achieved.

It is an object of the present invention to provide a general expression vector system giving high yields for the desired protein, in particular recombinant human G-CSF (rhG-CSF).

It is a further object of the present invention to provide stable eukaryotic cell lines containing multiple copies of the vector integrated into the host cell genomic DNA.

Summary of the invention

It has now been found that high levels of eukaryotic protein production can be achieved, steadily and for a wide variety of heterologous proteins, in CHO cells using an expression vector combining a number of advantageous genetic components. Surprisingly, it has been observed that the yields of eukaryotic protein obtained by using the expression vector according to the present invention, greatly exceed those obtained by using other contemporary expression systems. This result could not be

foreseen by considering the known properties of the individual components used in the claimed vector construction.

The recombinant DNA construct according to the present invention is useful for obtaining a high level of eukaryotic gene expression in the CHO dhfr$^-$ cell line, and comprises at least the following features:

- a strong eukaryotic promoter [preferably from cytomegalovirus (CMV)] upstream of a DNA sequence encoding the desired protein; as used hereinafter "promoter" comprises both the promoter itself (nucleotide sequences recognized by RNA-polymerase molecules starting RNA synthesis) and the promoter/enhancer combination (enhancer refers to cis-acting nucleotide sequences which act on a promoter to increase its transcriptional efficiency), whereas, in case the enhancer sequences are suitably deleted, that will be clearly indicated;

- a sequence for processing the RNA transcripts comprising the second intron and the beta-globin gene polyadenylation site; these sequences facilitate the transport from nucleus to cytoplasm and the translation of the mRNA;

- a marker sequence for selection and amplification; in this sequence the SV40 early region promoter is suitably modified in order to remove the SV40 enhancer (this has the effect of reducing the DHFR transcription so that efficient gene amplification can be obtained in CHO dhfr$^-$ cells). In addition, said sequence, hereinafter named SV1DHFR, contains the SV40 small-t antigen intron and the SV40 early region polyadenylation site to facilitate the transport from nucleus to cytoplasm and the translation of mRNA encoding the DHFR enzyme.

Furthermore, the eukaryotic expression vector according to the present invention contains a DNA fragment, derived from pSV2dhfr, containing the origin of replication and ampicillin resistence gene derived from pBR322.

This facilitates replication and selection of E.coli transformants.

Moreover, the vector according to the present invention contains a DNA sequence encoding the desired protein. Preferably, said vector contains a cDNA, encoding an eukaryotic protein of interest, which has been modified in order to remove the sequences responsible for the destabilization of the corresponding mRNA. Such sequences are located in the 3' untranslated region of the mRNA and contain repeats of the AUUUA motif which is believed to be responsible for destabilization of short-lived mRNA (Shaw and Kamen, Cell, 46, 659-667, 1986).

The murine cytomegalovirus (MCMV) major immediate early promoter is preferably used (Dorsch-Hasler et al., Proc. Natl. Acad. Sci., USA, 82, 8325-8329, 1985).

The second intron and the polyadenylation site preferably derive from rabbit beta1-globin gene (Rohrbaugh et al., Mol. Cell. Biol. 5, 147-160, 1985).

The sequence used as marker is preferably a DNA fragment derived from the commercially available pSV2dhfr vector (ATCC 37146), and containing the dihydrofolate reductase (DHFR) cDNA coupled to the SV40 early promoter for expression in eukaryotic cells.

A further embodiment of the present invention provides a stably transformed CHO dhfr$^-$ cell line containing multiple copies (up to 2000 per cell) of the transfected sequences and able to synthesize the desired eukaryotic protein at high levels. Examples of eukaryotic protein expression include human granulocyte colony stimulating factor (hG-CSF) as well as human GM-CSF and human erythropoietin.

According to another aspect of the present invention, there is provided a method for producing the said eukaryotic protein which comprises culturing the previously described stably transformed CHO dhfr$^-$ cell

line, containing multiple copies of the expression vector, and recovering the said protein.

A further aspect of the present invention relates to the preparation of the vector using the human cytomegalovirus (HCMV) major immediate early promoter and then using the obtained vector in a suitable cell line [for example, CHOdhfr$^-$ or cells of the Hep G2 line (ATCC HB8065)].

Reference to drawings

Figure 1 illustrates plasmid pMCMVβ #51-2 containing the origin of replication (ORI) and ampicillin resistence gene (Amp R) of pBR322; the MCMV major immediate early promoter (comprised between AccI and HindIII); the sequence, for processing of the RNA transcripts, obtained from rabbit beta1-globin gene (comprised between SacI and BamHI) comprising the beta1-globin second intron, and polyadenylation site; and a multiple cloning site (polylinker) suitable for insertion of the desired DNA fragment encoding a desired heterologous protein.

Figure 2 illustrates plasmid pMCMV #53-2.

pMCMV #53-2 contains:

a) a 3.7 Kbp from AccI to Bg1II DNA fragment obtained from pSV2dhfr (ATCC 37146) containing the origin of replication and ampicillin resistence gene of pBR322, and the SV40 small-t antigen intron and the SV40 early region polyadenylation site;

b) the MCMV major immediate early promoter (a DNA fragment identical to that used in the pMCMVβ #51-2 vector construction);

c) a polylinker suitable for insertion of the desired DNA fragment encoding a desired heterologous protein. The polylinker was obtained by annealing the following two complementary synthetic DNA sequences reported in SEQ ID NO: 1 and SEQ ID NO: 2.

Figure 3 illustrates plasmid pMCMVβ G-CSF #55-4, derived from plasmid

pMCMVβ #51-2 by insertion of the sequence encoding hG-CSF.

Figure 4 illustrates plasmid pMCMV G-CSF #57-12 derived from plasmid pMCMV #53-2 by insertion of the sequence encoding hG-CSF.

Figure 5 illustrates plasmid pMCMVβ G-CSF-SV1-DHFR #69-1 derived from plasmid pMCMVβ G-CSF #55-4 by addition of the SV1DHFR transcription unit.

Figure 6a illustrates the preparation of plasmid pSV2-G-CSF.

Figure 6b illustrates the preparation of plasmid pSV2(G-CSF-dhfr).

Detailed description of the invention

The invention relates to a system for producing correctly processed and post-translationally modified eukaryotic proteins in CHO cells by using recombinant DNA technology. Preferably, these proteins are of human therapeutic or diagnostic value, such as growth Factors, lymphokines, interferons, enzymes or antigens. It will be remarked that although the production of mature proteins is preferred, hybrid proteins or protein fragments having the relevant activity may also be produced.

The pMCMVβ #51-2 DNA construct shown in Figure 1 contains, in addition to the 2.9 Kbp from AccI to BamHI DNA fragment obtained from pSV2dhfr (ATCC 37146), the origin of replication and ampicillin resistence gene of pBR322, and contains also the MCMV major immediate early promoter comprising nucleotides from -492 to +39 as described by Dorsch-Hasler et al. (Proc. Natl. Acad. Sci., USA, 82, 8325-8329, 1985). Such DNA fragment was obtained by PCR amplification using plasmid pON402 (mentioned in the reference of Manning and Mocarski, Virology, 167, 477-484, 1988) as the template. For the amplification, it was used a 5' primer containing the sequence SEQ ID NO:3 and a 3' primer of sequence SEQ ID NO:4 : the 5' and 3' primers contain respectively an AccI restriction site and a HindIII site in order to facilitate the vector preparation by using standard recombinant DNA technology (Maniatis et al., Molecular Cloning: A

Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1982). Plasmid pMCMVβ #51-2 also contains a polylinker obtained by annealing two synthetic complementary DNA sequences: SEQ ID NO:5 and SEQ ID NO:6.

The presence of this multiple cloning site facilitates subsequent insertion of the DNA fragment encoding the protein of interest. Plasmid pMCMVβ #51-2 also contains a DNA fragment containing the rabbit beta1-globin gene second intron and the polyadenylation site corresponding to nucleotides from +478 to +1633 using the nucleotide numbering system of Rohrbaugh et al (cited before). This fragment was obtained thanks to standard PCR technology using plasmid pUK4.7 as template. Plasmid pUK4.7 contains the 4.7 Kbp fragment of KpnI ends from rabbit beta1-globin gene (Lacy et al., Cell 18, 1273-1283, 1979) inserted in the KpnI site of plasmid pUC19 (kindly provided by courtesy of Dr. R. Hardison. The Pennsylvania State University, PA, USA).

The synthetic DNA primers used are reported in SEQ ID NO:7 and SEQ ID NO:8.

The former primer contains a SacI restriction site and the latter primer contains a BamHI restriction site in order to facilitate vector construction by using standard cloning technology.

Plasmid pMCMVβ #51-2 represents a basic eukaryotic expression system, having a strong promoter and enhancer derived from MCMV and the second intron splice site and polyadenylation signal derived from rabbit beta1-globin gene. It contains a polylinker to facilitate subsequent insertion of the fragment encoding the desired protein, as well as sites for the insertion of the transcription unit SV1DHFR derived from commercially available plasmid pSV2dhfr. It should be noted that the plasmid lacks an origin of replication for mammalian cells and thus it will not replicate

unless it becomes integrated into the mammalian chromosome. After that the plamid is integrated in a chromosome, the cells it transformed, which amplified the number of copies of said plasmid, are selected using increasing levels of methotrexate (MTX), a dihydrofolate reductase inhibitor. It is possible to obtain an amplification of about 2000 copies per cell and this results in a high efficient expression of the desired protein.

The vector using HCMV major immediate early promoter contains the nucleotides sequence from -737 to +116 (Boshart et al., Cell, 41, 521-530, 1985) which was amplified by PCR, using plasmid pRL103 as template. Said plasmid is described in Pizzorno et al., J. Virol., 62, 1167-1179, 1988). The PCR 5' primer includes a AccI site and has the sequence SEQ ID NO:9; the PCR 3' primer includes a HindIII site and has the sequence SEQ ID NO:10.

Some specific embodiments according to the present invention are now exemplified with reference to the drawings.

Example 1

Construction of a hG-CSF expression vector

The complete coding sequence of hG-CSF was amplified by standard PCR technology using plasmid pG-CSF6 (Tweardy et al, Oncogene Research 1, 209-220, 1987) as template. The sequence of the 5' PCR primer is reported in SEQ ID NO:11 and that of the 3' PCR primer is reported in SEQ ID NO:12. Both primers contain a XbaI site enabling the cloning of the PCR fragment, containing the hG-CSF coding sequence, into the XbaI site of pBluescript SK plasmid (Stratagene, La Jolla, CA, USA) to obtain plasmid pBS G-CSF. The analysis of the sequence amplified by PCR confirmed that the coding region was identical to that of Tweardy et al (Oncogene Research 1, 209-220, 1987).

It is known in the prior art (Shaw and Kamen; as above) that the mRNAs encoding many cytokines, proto-oncogenes, lymphokines and interferons are unstable due to the presence of a $(AUUUA)^n$ repeated motif, located in the 3'-untranslated region. In particular, it has been shown that the introduction of a 51-nucleotide sequence (derived from the 3' untraslated region of the hGM-CSF) rich in AT into the 3' untraslated region of the rabbit beta-globin gene caused the otherwise stable beta-globin mRNA to become highly unstable in vivo.

The authors of the present invention found that removing the 3' untraslated region of hG-CSF mRNA containing the AUUUA repeated motifs, gave a higher level of G-CSF protein production.

It is to be noted that the 3' untranslated region of the hG-CSF cDNA was removed by PCR amplification and that the two PCR primers contained a XbaI site for cloning into pBluescript.

The sequence encoding hG-CSF, free of the $(AUUUA)^n$ instability motifs, was then cloned into the unique XbaI site of plasmid pMCMVβ #51-2 (Figure 1) and of plasmid pMCMV #53-2 (Figure 2) resulting in plasmids pMCMVβ G-CSF #55-4 and pMCMV G-CSF #57-12 as respectively shown in Figures 3 and 4.

Example 2

Construction of a DHFR amplification vector

Plasmid pMCMVβ G-CSF #55-4 (Fig.3) lacks a selection marker for eukaryotic cells and this was provided by using the SV1DHFR transcription unit obtained from the commercially available plasmid pSV2dhfr. Said unit comprises the SV40 early region promoter (from which the enhancer has been removed by digestion with SphI), the DHFR cDNA, the SV40 small-t antigen intron and early region polyadenylation site. Deletion of enhancer sequences from SV40 early region promoter in order to reduce the

level of recombinant gene expression was previously described (Gorman et al, Mol. Cell Biol. 2, 1044-1051, 1982). The lack of an enhancer leads to inefficient transcription of the DHFR cDNA so that cells having multiple integrated copies of the DHFR gene are preferably selected by methotrexate (MTX) selection.

In order to obtain the here above cited SV1 DHFR transcription unit, the authors of the present invention isolated, from pSV2dhfr, the 1.8 Kbp from SphI to BamHI fragment containing an enhancerless SV40 early promoter, the DHFR cDNA and the SV40 small-t antigen intron and polyadenylation site. This fragment was cloned into the pUC131 vector, between the SphI and BamHI sites. This cloning step positioned a new EcoRV site next to the SphI site and both a SalI and a PstI site next to the BamHI site.

In a second step, the DHFR transcription unit was isolated as EcoRV to PstI end fragment and cloned into the pUC130 vector, between HincII and PstI sites. From the last construct the DHFR transcription unit, named SV1DHFR, can be isolated as a BamHI fragment.

Plasmids pUC130 and pUC131 were constructed by replacing the PvuII fragment into pUC8 with that containing the polylinker from M13tg130 or M13tg131, respectively (Kieny et al, Gene 26, 91-99, 1983).

The SV1DHFR transcription unit contained between two BamHI sites was ligated to plasmid pMCMVβ CSF #55-4 which has also been BamHI cleaved, as shown in Fig.5. The resulting plasmid pMCMVβ G-CSF-SV1DHFR #69-1 (Fig.5) contains all the elements necessary for integration, selection, amplification, and high rhG-CSF expression.

Example 3

Production of stable CHO cell lines expressing rhG-CSF

The CHO host cell is a CHO dhfr$^-$ cell line described by Urlaub G. and

Chasin L.A., PNAS 77:4216-4220, 1980 and obtained from Dr. P.Reddy at the Wistar Institute Philadelphia, PA, USA.

CHOdhfr$^-$ cells were transformed by transfection with plasmid pMCMVβ G-CSF-SV1DHFR #69-1 (Fig.5) and dhfr$^+$ transformants were selected in nucleoside free medium.

Secreting rhG-CSF stable transformants in which amplification of the transfected sequences occurred were selected in a nucleoside free mediun containing MTX at concentration 160-320 mM; MTX resistant clones were subsequently isolated and some of them shown to express rhG-CSG at higher levels, compared to those shown by the transformants previously selected in the nucleoside free medium.

Example 4

Production of rhG-CSF by stably transformed CHO cell lines

The ability of individual clones, selected in the nucleoside free medium, to produce rhG-CSF and to export it into the medium was investigated by a hG-CSF ELISA test (Quantikine^{TM} human G-CSF Immnoassay #DCS00 R&D Systems Inc.).

The results are shown in Table 1.

Table 1

Clone	Productivity µg rhG-CSF/10^6 cells/24h
A 3-1	13
A 3-3	11
A 3-5	21
A 3-8	9.5

Two clones were chosen and the production of rhG-CSF measured on microcarrier-spinner culture. In order to settle the optimum growth time wherein cultures achieve the optimal protein yield, trials have been conducted and, in the medium in which cells were grown (conditioned

medium), the desired protein was assayed in a time course experiment. The results are shown in Table 2.

Table 2

Clone	Time h	Productivity mg/l
A 3-3	5	3.5
A 3-3	8	4.3
A 3-3	24	10.5
A 3-5	5	3.5
A 3-5	8	5.4
A 3-5	24	10.3

The identity of the expressed protein was confirmed by radioimmune precipitation assays (Protocol as in Sambrook et al., Molecular Cloning a Laboratory Manual, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1989) and the molecular weight was estimated to be 20KD by polyacrylamide gel electrophoresis followed by autoradiography.

In transient expression experiments performed in CHO dhfr$^-$ cells using supercoiled plasmid DNA, the MCMV promoter-based vectors (i.e. pMCMVβ G-CSF #55-4, pMCMV G-CSF #57-12, pMCMVβ G-CSFSV1DHFR #69-1) as claimed her, allow a G-CSF productivity of about 6-100 times higher than that obtained with the SV40 early promoter-based vector, the pSV2 (G-CSF-dhfr) as described in Table 3. Said vector was constructed by fusing parts of two vectors; pSV2neo (ATCC 37149) and pSV2dhfr (Subramani et al, Mol. Cell Biol. 1, 854-864, 1981). In pSV2 (G-CSF-dhfr) the hG-CSF cDNA from pG-CSF6 plasmid (Tweardy et al), contains in the 3' untranslated region AU destabilizing sequences, is under the transcriptional control of SV40 early region promoter and uses processing signals for transcripts from SV40 (SV40 small-t antigen intron and early region polyadenylation site). The vector pSV2 (G-CSF-dhfr) is the nearest if related to the constructs

described in the prior art for G-CSF expression. It should be noted, moreover, that since plasmid pMCMVβ G-CSF #55-4 and pMCMV #57-12 both contain: 1) the same promoter; 2) the same cDNA of the G-CSF gene deleted of destabilizing sequences; and 3) signals for 3' transcripts processing (intron and polyadenylation site from beta-globin and SV40, respectively) it is surprising the 10-times difference in the efficiency of expression observed between the two vectors mentioned above.

Table 3 shows the unpredictable increase of rhG-CSf yield obtained when using vector pMCMVβ G-CSF #55-4(and vector pMCMVβ G-CSFSV1DHFR #69-1).

Table 3

rhG-CSF ng/1x10^6 cells/24 h

Construct	Exp.1 (2µg DNA/dish)	Exp.2 (2µg DNA/dish)	Exp.3*
pMCMVβ G-CSF #55-4	>370	81	99
pMCMV G-CSF #57-12	65	5	10.5
pMCMVβ G-CSF SV1DHFR #69-1	n.d.	n.d.	115.5
pSV2 (G-CSF dhfr)	11	0.25	1.65

The results are the average of an experiment performed in duplicate. * The experiment has been carried out using 2µg of pMCMVG-CSF #57-12 DNA/dish and equimolar amounts of the indicated plasmid DNA. n.d.= not determined

CHOdhfr$^-$ cells were cultivated in MEM alpha medium containing nucleotides and deoxyribonucleosides (Gibco BRL), supplemented with 5% fetal calf

serum. Cells were plated in a 60mm dish and were transfected with the indicated amount of plasmid DNA using the cationic lipid Lipofectin^R Reagent (Gibco BRL) according to the manifacture's instruction. Cells were left in contact with the Lipofectin^R Reagent-DNA complex in 3 ml of serum-free medium (MEM alpha medium containing nucleosides and deoxyribonucleosides) for 6 hours, after which 3 ml of the medium supplemented with 10% fetal calf serum were added. Medium was replaced by the standard culture medium 24 hours after transfection. The medium in which cells were grown between 24 and 48 hours after transfection was tested by the R&D System Inc. hG-CSF ELISA test.

Surprisingly the experiment shows that vectors pMCMVG-CSF #57-12 and 93/4-1 are equally efficient in the expression of rhG-CSF. In this case removing the mRNA destabilizing sequences is not sufficient in order to increase the expression level of the cytokine.

In case of vectors pMCMVβG-CSF #55-4 and 93/4-1 higher levels of expression are obtained with the plasmid pMCMVβG-CSF #55-4 not containing the mRNA destabilizing sequences showing that the removal of these sequences is a necessary step in order to obtain an efficient expression system.

As a whole these data show that high levels of eukaryotic recombinant protein synthesis can not be achieved only by removing the mRNA destabilizing sequences and that it is unpredictable the efficacy of recombinant DNA systems combining different advantageous genetic components.

Example 5

Construction of plasmids 93/2-9 and 93/4-1 containing mRNA destabilizing sequences)

From the plasmid pG-CSF6 (Tweardy et al.) we isolated the 0.21 kbp NaeI

to StuI fragment of the hG-CSF cDNA. The fragment includes part of the coding sequence for hG-CSF and 3' untraslated region containing the ATTTA repeated motif.

This fragment was cloned between the NaeI and SmaI sites of pMCMVβG-CSF #55-4 and pMCMVG-CSF #57-12 (both deleted by digestion of the 0.13 kbp NaeI to SmaI fragment) obtaining the vectors 93/2-9 and 93/4-1, respectively.

The vectors 93/2-9 and 93/4-1 contain a hG-CSF cDNA which includes the complete coding sequence and ATTTA repeated motif (mRNA destabilizing sequences) under the transcriptional control of the MCMV major immediate early promoter and use sequences for processing the RNA transcripts either from rabbit beta1-globin gene or SV40, respectivvely.

Table 4 shows the hG-CSF yields obtained in transient expression experiments performed in CHOdhfr$^-$ cells using supercoiled plasmid DNA of pMCMVβG-CSF #55-4, pMCMVG-CSF #57-12, 93/2-9 and 93/4-1.

Table 4

Construct (2µg plasmid DNA/dish)	ng rhG-CSF/1x10^6 cells/24 h
pMCMVβG-CSF #55-4	244
pMCMVG-CSF #57-12	36
93/2-9	51
93/4-1	39

The results are the average of an experiment performed in triplicate. CHOdhfr$^-$ cells were cultivated in MEM alpha medium containing nucleotides and deoxyribonucleosides (Gibco BRL), supplemented with 5% fetal calf serum. Cells were plated in a 60mm dish and were transfected with the

indicated amount of plasmid DNA using the cationic lipid Lipofectin^R Reagent (Gibco BRL) according to the manifacture's instruction. Cells were left in contact with the Lipofectin^R Reagent-DNA complex in 3 ml of serum-free medium (MEM alpha medium containing nucleosides and deoxyribonucleosides) for 6 hours, after which 3 ml of the medium supplemented with 10% fetal calf serum were added. Medium was replaced by the standard culture medium 24 hours after transfection. The medium in which cells were grown between 24 and 48 hours after transfection was tested by the R&D System Inc. hG-CSF ELISA test.

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As a whole these data show that high levels of eukaryotic recombinant protein synthesis can not be achieved only by removing the mRNA destabilizing sequences and that it is unpredictable the efficacy of recombinant DNA systems combining different advantageous genetic components.

Example 6

Purification of rhG-CSF from culture (conditioned) medium

A conditioned medium from CHO cells engineered with vector pMCMVβ G-CSF SV1DHFR #69-1, secerning rhG-CSF and grown up in microcarrier-spinner cultures, was filtered, adjusted to pH 7.6 with HCl and diluted 1:2 with

water. The purification proceeded in three column chromatography steps:

1) The sample was loaded on a Q-Sepharose FF column (16-200mm) previously equilibrated with 20mM Tris-HCl, pH 7.6. rhG-CSF was eluted with 0-0.5M sodium chloride linear gradient, in 20mM Tris-HCl buffer, pH 7.6.

2) The fractions containing rh-G-CSF were collected and loaded onto a Toyopearl Butyl 650S hydrophobic interaction column, previously equilibrated with 1.0M ammonium sulfate in 50mM phosphate buffer, pH 5.3; rhG-CSF was then eluted in gradient mode with 50mm phosphate buffer, pH 5.3, containing 50% 2-propanol.

3) The fractions containing rhG-CSf were collected and finally loaded onto a Pharmacia Sephacryl S 200 gel permeation column, previously equilibrated with a 20mM phosphate buffer pH 7.2, containing 50mM NaCl. The permeated fractions containing rhG-CSF were collected.

rhG-CSF samples were analysed by standard technology using reverse phase HPLC analysis and by a Western blot using the commercially available anti-hG-CSF polyclonal antibody (Genzyme).

The NH2-terminal amino acid sequence of purified rhG-CSF has been determined. The primary structure analysis of rhG-CSf was performed using a protein sequencer (Millipore Mod.6625). As a result, the amino acid sequence of 14 residues beginning from the NH2-terminus was determined and it is reported in SEQ ID NO:13.

The purified rhG-CSF product was tested for the biological activity according to the known techniques. The obtained results were considered of interest.

G-CSF (preferably hG-CSF) according to the present invention is used as active principle, optionally in combination with pharmaceutical acceptable carriers and excipients, for the preparation of suitable pharmaceutical compositions, e.g. for promoting hematopoiesis, for the

augmentation of the defence mechanism against infections, etc.

The present invention also relates to a pharmaceutical composition comprising as active principle a therapeutically effective amount of G-CSF (preferably hG-CSF) according to the present invention, optionally in combination with suitable pharmaceutically acceptable carriers and excipients.

The authors of the present invention have eventually found that G-CSF may be used directly for the treatment of tumors.

According to a preferred embodiment, hG-CSF is used as active principle, in association with a specific carrier specific for tumoral cells, for preparing an effective pharmaceutical composition in anti-tumor theraphy.

Claims

1. A vector system for the expression of a protein in a mammalian host cell, said vector system comprising at least the following elements:
- a CMV promoter upstream of a DNA sequence encoding the desired protein;
- a sequence for processing the RNA transcripts; and
- a marker sequence for the selection and amplification.

2. A vector system according to claim 1, characterized in that the CMV promoter is the MCMV major immediate early promoter.

3. A vector system according to claim 1, characterized in that the CMV promoter is the HCMV major immediate early promoter.

4. A vector system according to claim 1, characterized in that the sequence for processing the RNA transcripts comprises the beta-globin gene second intron and polyadenylation site.

5. A vector system according to claim 4, characterized in that the beta-globin gene is rabbit beta1-globin gene.

6. A vector system according to claim 1, characterized in that the marker sequence is the SV1DHFR transcription unit.

7. A vector system according to claims 1-6, characterized in that the DNA sequence encoding the desired protein is a cDNA sequence.

8. A vector system according to claims 1-7, characterized in that the protein to be expressed is G-CSF.

9. A vector system according to claim 8, characterizzed in that the DNA sequence encoding G-CSF has a deletion of the sequences responsible for the destabilization of mRNA transcripts.

10. A vector system according to claims 8-9, characterized in that the protein to be expressed is hG-CSF.

11. A process for the recombinant preparation of a protein by expression in a mammalian host cell, characterized in that said mammalian cell is

transformed or transfected by a vector system according to claims 1-10, cultivating said mammalian cells and recovering said protein.

12. A process according to claim 11, characterized in that the expression is carried out in CHO dhfr$^-$ cells.

13. A pharmaceutical composition comprising as active principle an effective therapheutically amount of hG-CSF according to claims 8-10, optionally in combination with pharmaceutically acceptable carrier and excipients.

14. Use of hG-CSF according to claims 8-10 as active principle for the preparation of a pharmaceutical composition in combination with a carrier and an excipient.

15. Use of hG-CSF according to claims 8-10 active principle, in combination with a suitable carrier specific for tumoral cells, for the preparation of a pharmaceutical composition useful in anti-tumor theraphy.

FIG.1

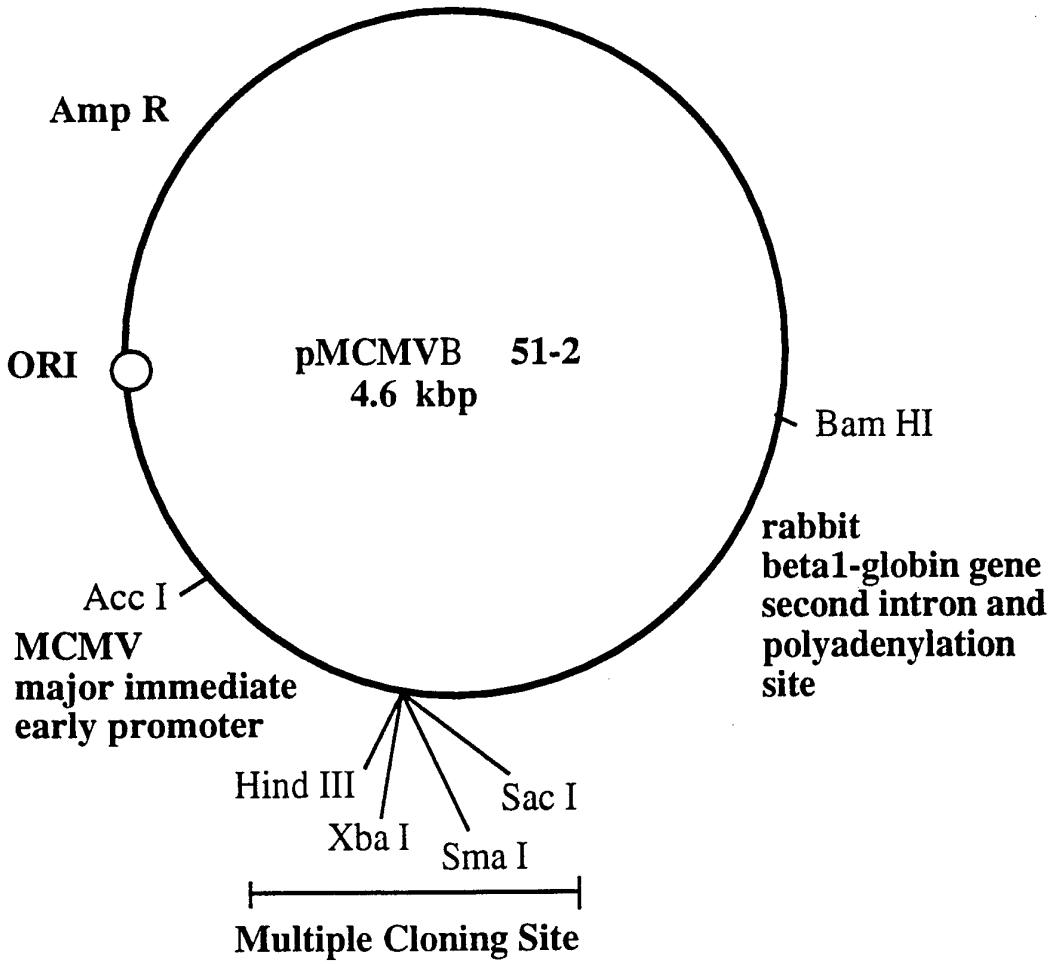
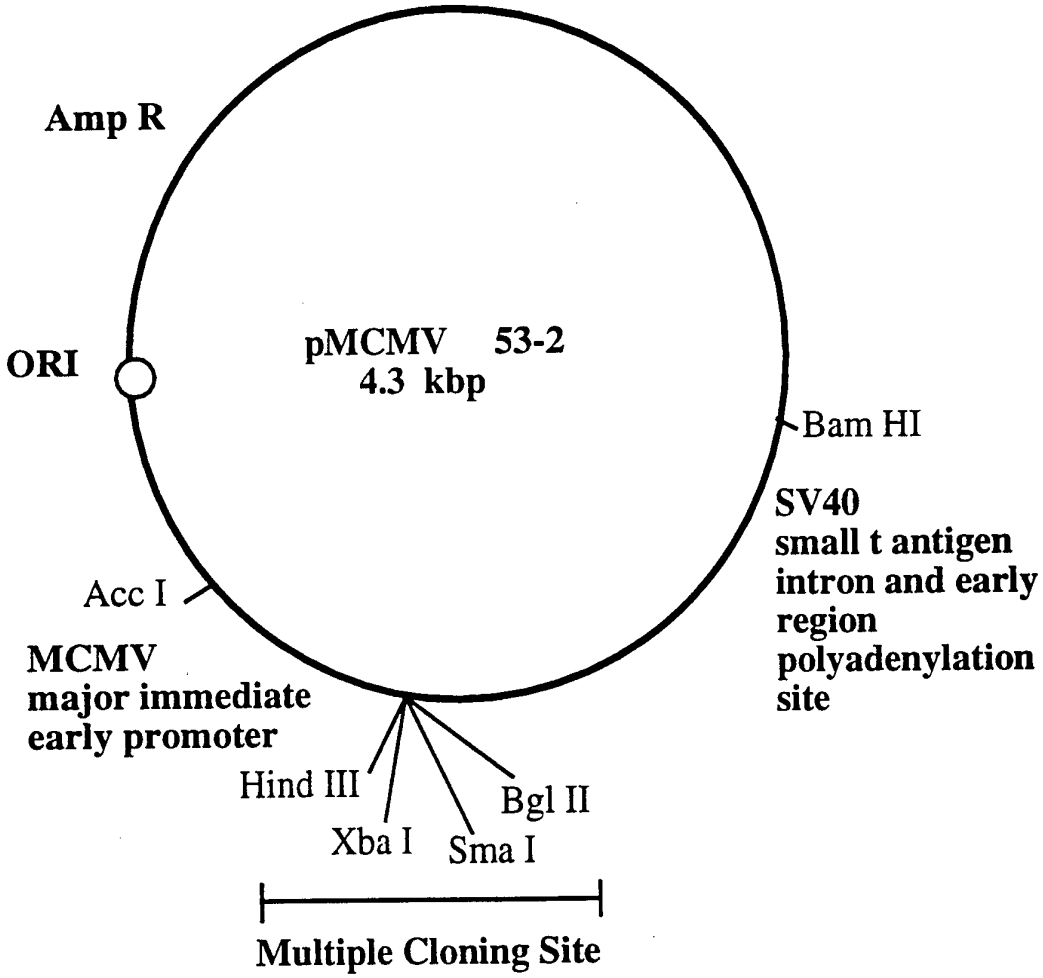

Amp R
ORI
pMCMVB 51-2
4.6 kbp
Bam HI
rabbit
beta1-globin gene
second intron and
polyadenylation
site
Acc I
MCMV
major immediate
early promoter
Hind III
Xba I
Sma I
Sac I
Multiple Cloning Site

FIG. 2


Amp R
ORI
pMCMV 53-2
4.3 kbp
Bam HI
SV40
small t antigen
intron and early
region
polyadenylation
site
Acc I
MCMV
major immediate
early promoter
Hind III
Xba I
Sma I
Bgl II
Multiple Cloning Site

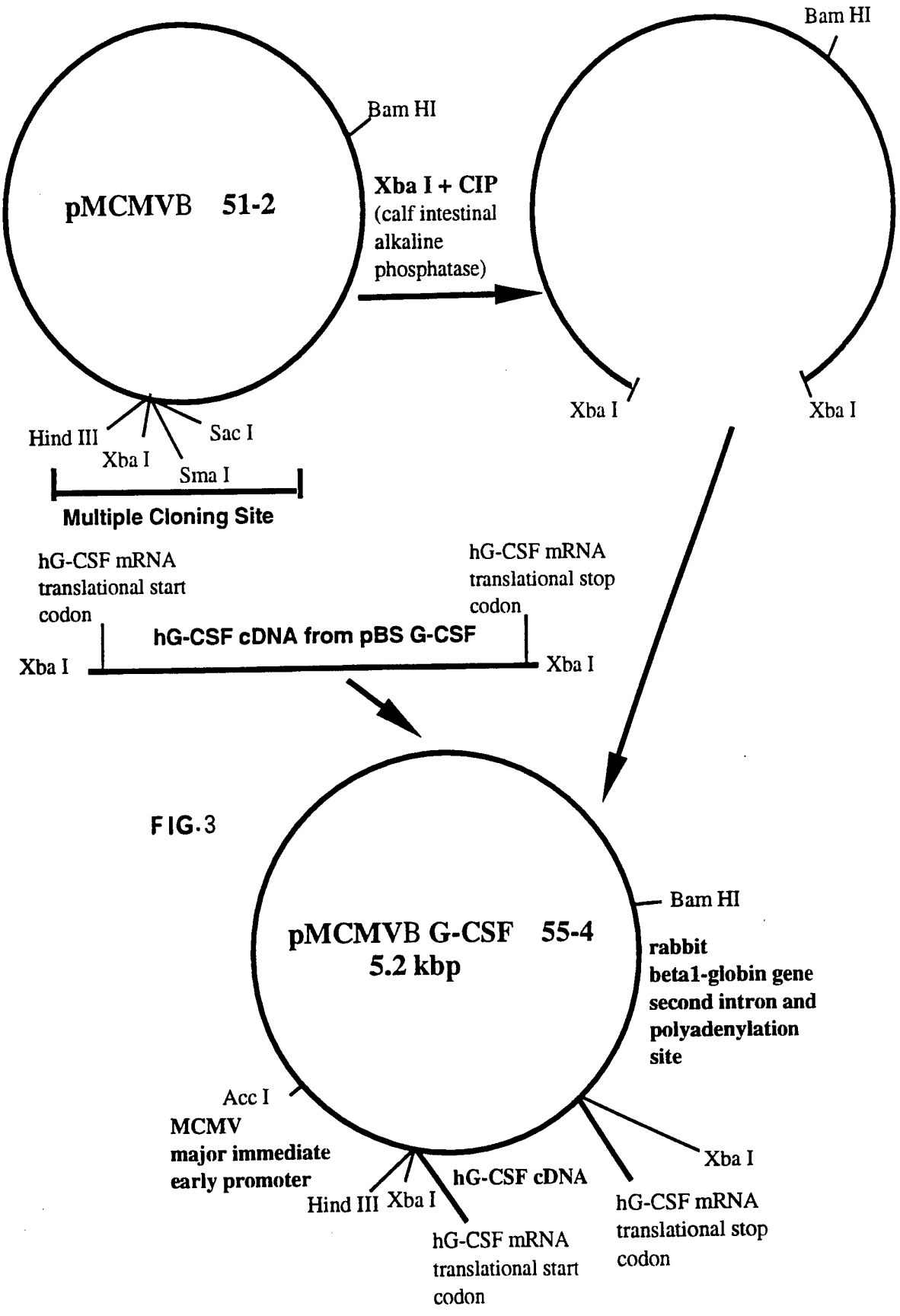

Bam HI
pMCMVB 51-2
Xba I + CIP
(calf intestinal
alkaline
phosphatase)
Bam HI
Xba I
Xba I
Hind III
Xba I
Sac I
Sma I
Multiple Cloning Site
hG-CSF mRNA
translational start
codon
hG-CSF cDNA from pBS G-CSF
hG-CSF mRNA
translational stop
codon
Xba I
Xba I
pMCMVB G-CSF 55-4
5.2 kbp
Bam HI
rabbit
beta1-globin gene
second intron and
polyadenylation
site
Acc I
MCMV
major immediate
early promoter
Hind III
Xba I
hG-CSF cDNA
hG-CSF mRNA
translational start
codon
Xba I
hG-CSF mRNA
translational stop
codon

FIG.3

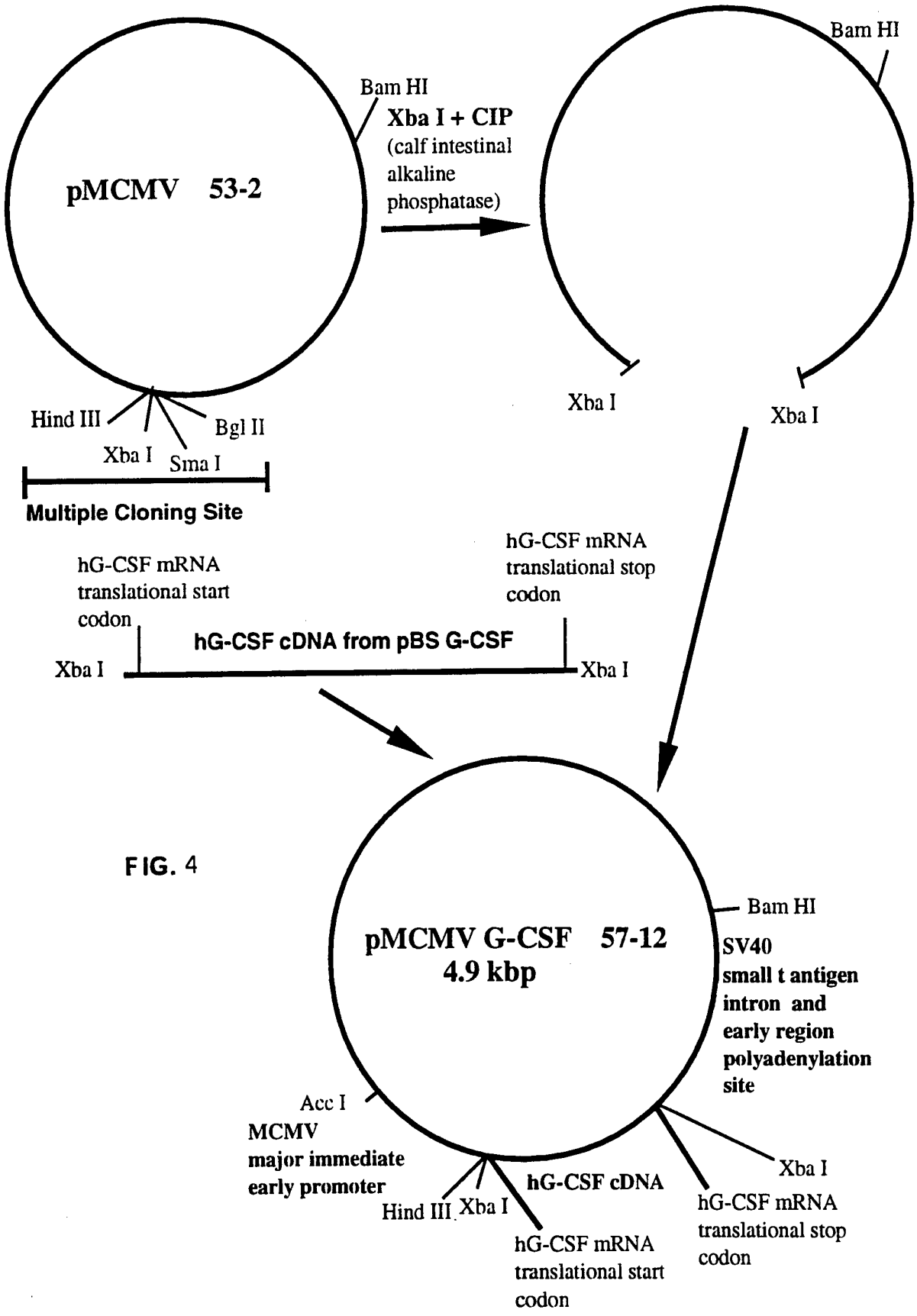
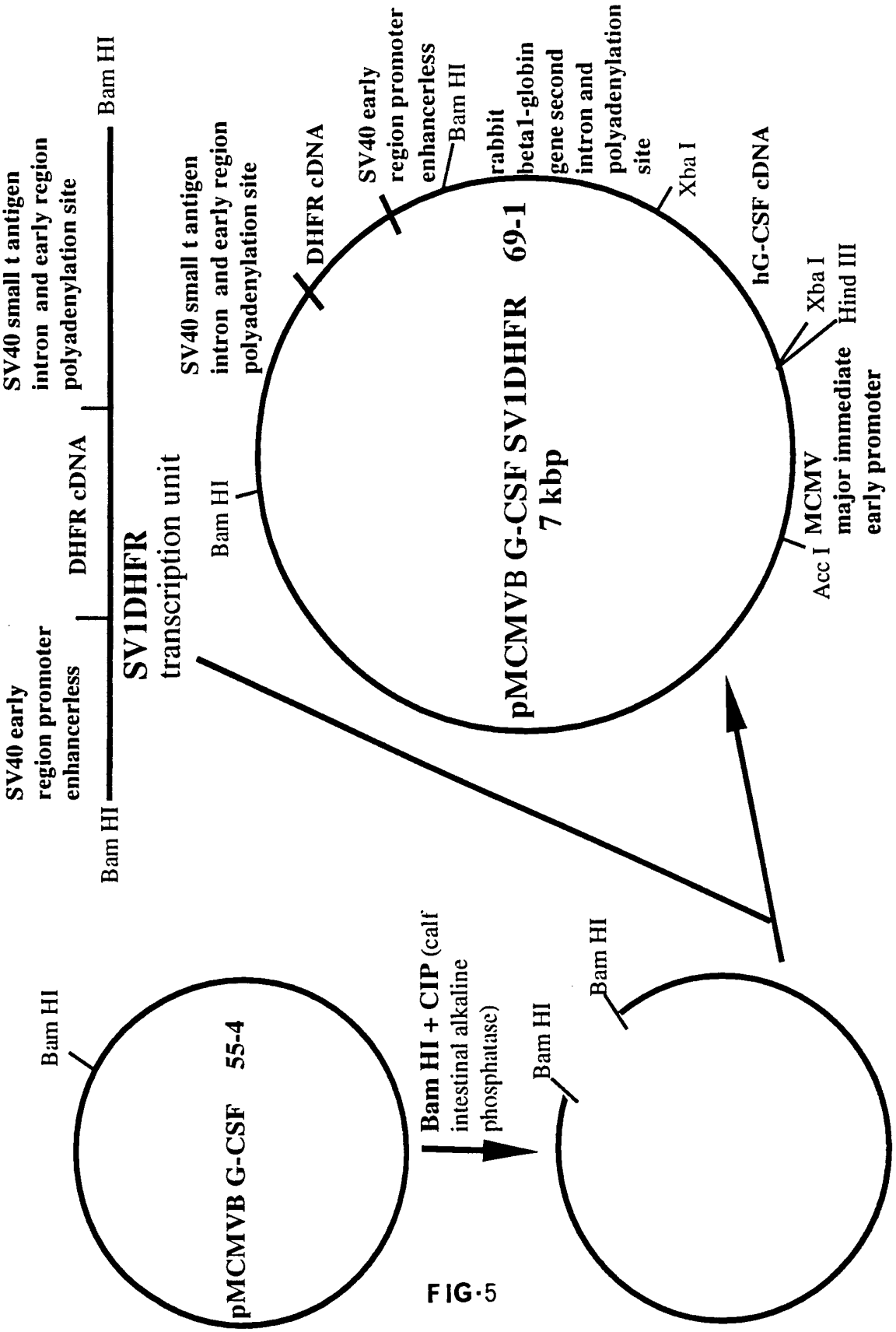

pMCMV 53-2
Bam HI
Xba I + CIP
(calf intestinal
alkaline
phosphatase)
Bam HI
Xba I
Xba I
Hind III
Xba I
Sma I
Bgl II
Multiple Cloning Site
hG-CSF mRNA
translational start
codon
hG-CSF mRNA
translational stop
codon
hG-CSF cDNA from pBS G-CSF
Xba I
Xba I
pMCMV G-CSF 57-12
4.9 kbp
Bam HI
SV40
small t antigen
intron and
early region
polyadenylation
site
Acc I
MCMV
major immediate
early promoter
Hind III
Xba I
hG-CSF cDNA
hG-CSF mRNA
translational start
codon
Xba I
hG-CSF mRNA
translational stop
codon

FIG. 4


Bam HI
pMCMVB G-CSF 55-4
Bam HI + CIP (calf intestinal alkaline phosphatase)
Bam HI
Bam HI
SV40 early region promoter enhancerless
DHFR cDNA
SV40 small t antigen intron and early region polyadenylation site
Bam HI
SV1DHFR transcription unit
Bam HI
SV40 small t antigen intron and early region polyadenylation site
DHFR cDNA
SV40 early region promoter enhancerless
Bam HI
rabbit beta1-globin gene second intron and polyadenylation site
Xba I
hG-CSF cDNA
Xba I
Hind III
MCMV major immediate early promoter
Acc I
pMCMVB G-CSF SV1DHFR 69-1
7 kbp

FIG·5

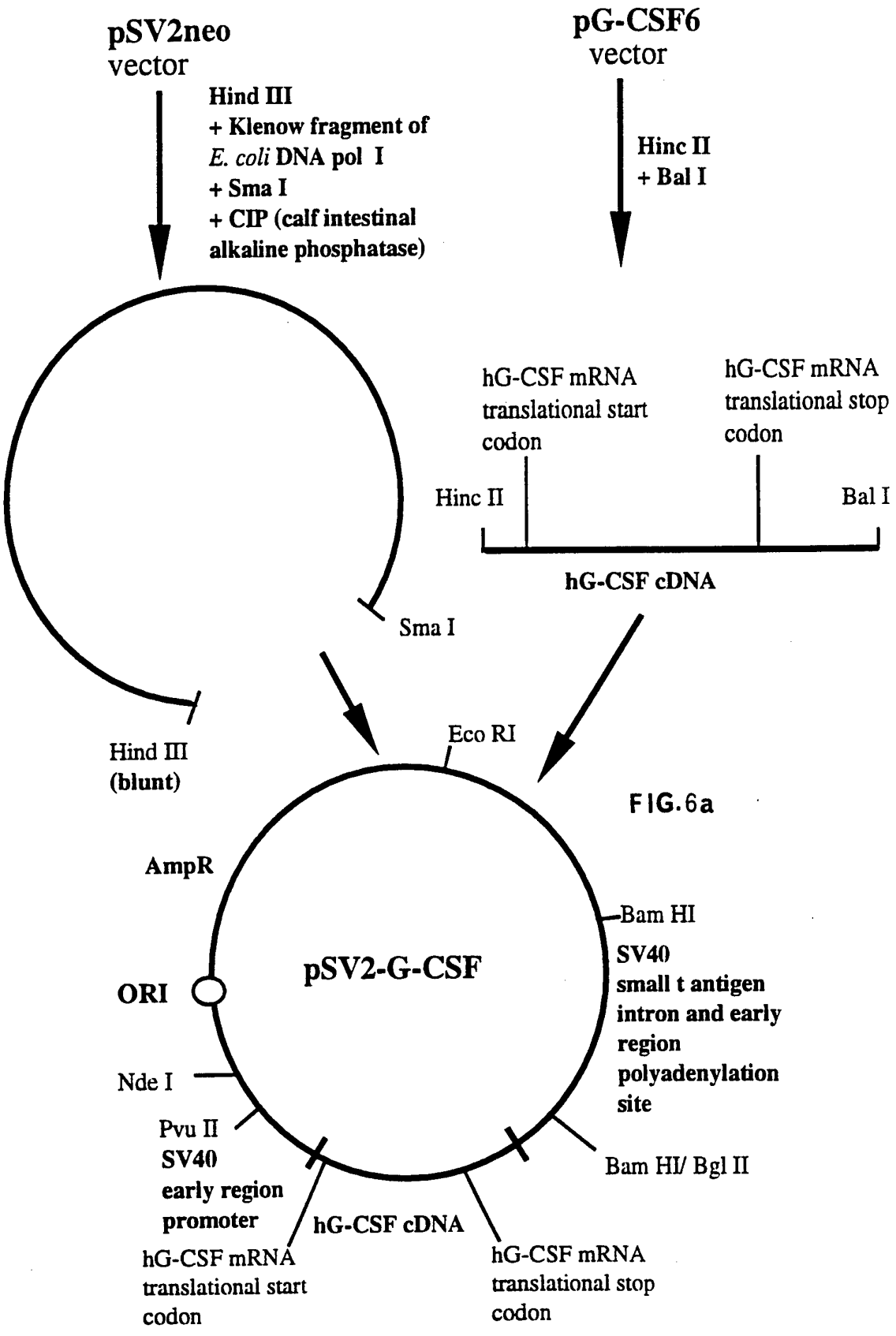
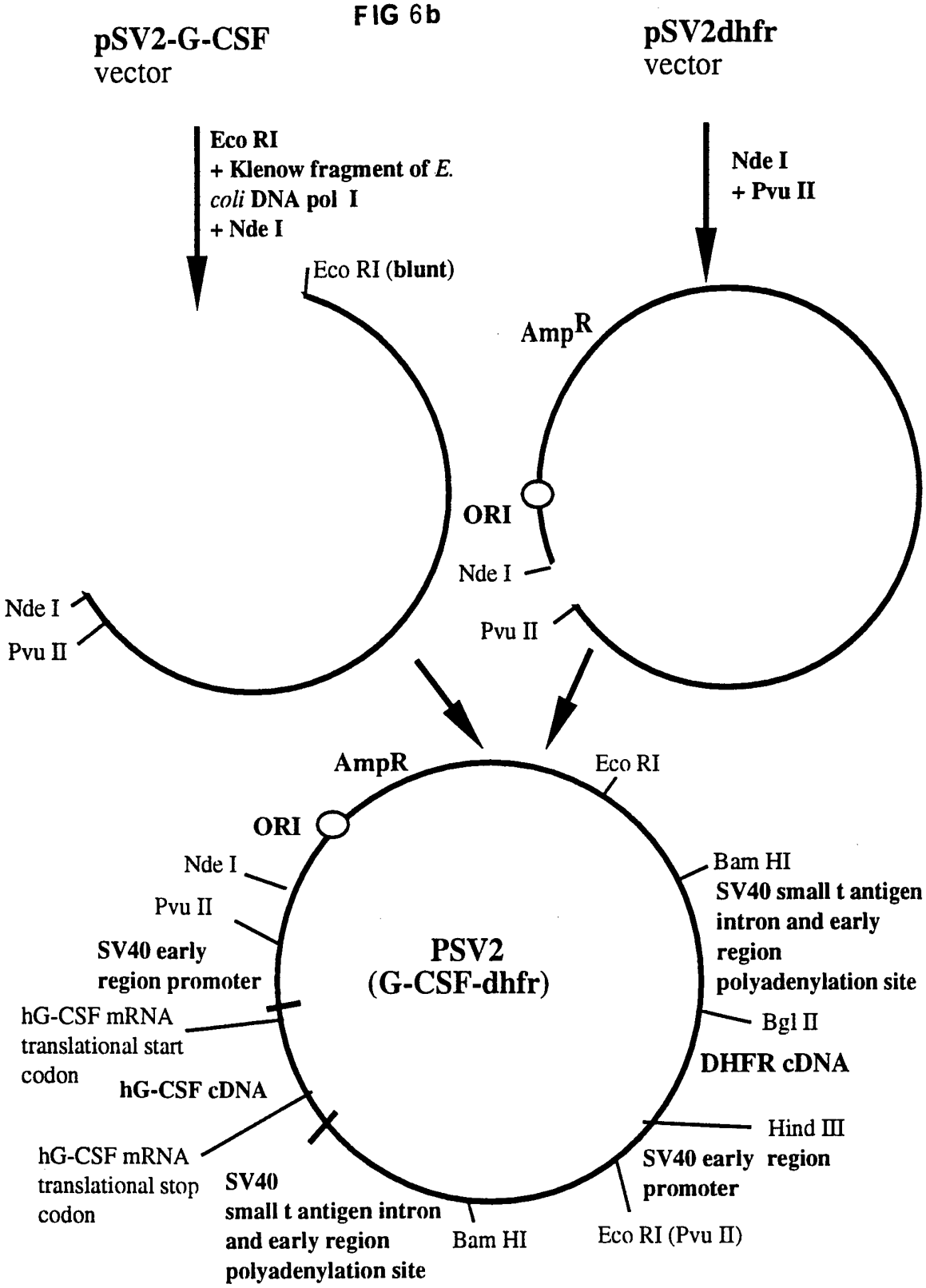

pSV2neo
vector
Hind III
+ Klenow fragment of
E. coli DNA pol I
+ Sma I
+ CIP (calf intestinal
alkaline phosphatase)
pG-CSF6
vector
Hinc II
+ Bal I
hG-CSF mRNA
translational start
codon
hG-CSF mRNA
translational stop
codon
Hinc II
Bal I
hG-CSF cDNA
Sma I
Hind III
(blunt)
Eco RI
AmpR
Bam HI
pSV2-G-CSF
SV40
small t antigen
intron and early
region
polyadenylation
site
ORI
Nde I
Pvu II
SV40
early region
promoter
Bam HI/ Bgl II
hG-CSF cDNA
hG-CSF mRNA
translational start
codon
hG-CSF mRNA
translational stop
codon

FIG.6a

FIG 6b


pSV2-G-CSF
vector
pSV2dhfr
vector
Eco RI
+ Klenow fragment of E. coli DNA pol I
+ Nde I
Nde I
+ Pvu II
Eco RI (blunt)
AmpR
ORI
Nde I
Pvu II
Nde I
Pvu II
AmpR
Eco RI
ORI
Nde I
Pvu II
SV40 early region promoter
hG-CSF mRNA translational start codon
hG-CSF cDNA
hG-CSF mRNA translational stop codon
SV40 small t antigen intron and early region polyadenylation site
Bam HI
Eco RI (Pvu II)
PSV2
(G-CSF-dhfr)
Bam HI
SV40 small t antigen intron and early region polyadenylation site
Bgl II
DHFR cDNA
Hind III
SV40 early region promoter

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 94/03488

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12P21/02 A61K38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANALYTICAL BIOCHEMISTRY, vol. 209, 1993 pages 327-331, FRED A. M. ASSELBERGS ET AL. 'A two-plasmid system for transient expression of cDNAs in primate cells' *see the whole document* ---	1,2,4,5, 7,11
Y	IDEM, *see the whole document* ---	3,8-10, 12-15
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 March 1995	20 -03- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Marie, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ONCOGENE RESEARCH, vol. 1, 1987 pages 209-220, DAVID J. TWEARDY ET AL. 'Molecular cloning and characteritation of a cDNA for human G-CSF...' *see the whole document* ---	8-10, 13-15
Y	CELL, vol. 46, 1986 pages 659-667, GRAY SHAW ET AL. 'A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediastes selective mRNA degradation' *see the whole document* ---	9
Y	WO,A,89 01036 (CELLTECH LIMITED) 9 February 1989 *see the whole document* ---	3
Y	EP,A,0 265 874 (THE GREEN CROSS CORPORATION) 4 May 1988 *see the whole document* -----	12

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 94/03488

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8901036	09-02-89	DE-A-	3880468	27-05-93
		EP-A,B	0323997	19-07-89
		JP-T-	2500330	08-02-90
EP-A-0265874	04-05-88	JP-A-	63105675	10-05-88