EXPRESSION OF GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR GENE IN INSECT CELLS BY A BACULOVIRUS VECTOR

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

BY

Chuang-Jiun Chiou, B.S.

Denton, Texas

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The Baculovirus Expression Vector System (BEVS) has been used to express more than 50 different genes. Recombinant proteins have been produced at levels ranging from 1 mg/L to 600 mg/L. The focus of this research is to describe the production and characterization of the human granulocyte-macrophage colony-stimulating factor (hGM-CSF) in insect cells, using *Autographa californica* nuclear polyhedrosis virus (AcNPV) as an expression vector. To construct the vAc373GM-CSF recombinant virus, the hGM-CSF cDNA was inserted into the baculovirus polyhedrin plasmid pAc373. A new construct (pAc373GM-CSF) was obtained which contains the hGM-CSF gene in the proper orientation downstream of the strong polyhedrin promoter. Supercoiled pAc373GM-CSF DNA was subsequently cotransfected with wild-type AcNPV viral DNA into cell lines from the Fall Army worm *Spodoptera frugiperda* (Sf9). *In vivo* recombination events yielded a small population of recombinant viruses with the hGM-CSF sequences incorporated into the original viral genome. This vAc373GM-

CSF chimeric viral isolate was identified and purified through a procedure involving successive rounds of plaque hybridization. By assaying the culture medium, it was demonstrated that recombinant virus infected Sf9 cells expressing hGM-CSF which stimulates colony formation from human progenitor cells. Recombinant hGM-CSFs with apparent molecular weights of 14.5, 15.5 and 16.5 KDa were detected in infected culture medium by the Western blot method. All three forms have the biological activity of hGM-CSF. Following N-glycanase treatment, the two glycosylated hGM-CSF proteins (15.5 and 16.5 KDa) which bound to Concanavalin A affinity column ran as a 14.5-15.5 KDa band on SDS-PAGE. Western blot analysis of expression in Sf9 cells treated with tunicamycin revealed only the presence of the 14.5 KDa species. The N-terminal amino acid sequence of the recombinant hGM-CSF was identical to that of natural hGM-CSF deduced from cDNA. These results demonstrate that baculovirus-produced hGM-CSF could be N-glycosylated in Sf9 cells, the signal peptide of recombinant hGM-CSF could be recognized and cleaved by infected insect cells and the resultant molecule secreted into the medium.

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Abbreviations

AcNPV	*Autographa californica* nuclear polyhedrosis virus
AIDS	Acquired immunodeficiency syndrome
Ap	Ampicillin
ATP	Adenosine 5'-triphosphate
bp	Base pair(s)
BSA	Bovine serum albumin
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
cDNA	Complementary deoxyribonucleic acid
CM	Conditioned medium
COS	CV-1 monkey cell line
CSF	Colony-stimulating factor
ddH_2O	Double distilled water
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DNA PolI	Deoxyribonucleic acid polymerase I
DME medium	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
E.coli	*Escherichia coli*
ECV	Extracellular virus
EDTA	Ethylenediaminetetracetic acid
EtBr	Ethidium bromide (2,7-Diamino-10-ethyl-9-

	phenylphenanthridinium bromide)
FCS	Fetal calf serum
GAR-HRP	Goat anti-rabbit-horseradish peroxidase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Haemagglutinin
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hpi	Hour(s) post infection
HTLV	Human T-cell leukemia virus
IL-3	Interleukin 3
Kb	Kilobases or kilobasepairs
LB medium	Per liter
	Bacto-tryptone 10 gm
	Bacto-yeast extract 5 gm
	NaCl 10 gm
M-CSF	Macrophage colony-stimulating factor
Mo	HTLV-transformed T-lymphoblast cell line
M.O.I.	Multiplicity of infection
Mr	Apparent molecular weight
Occ^-	Occlusion negative virus
Occ^+	Occlusion positive virus
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol

pfu	Plaque forming unit
PHA	Phytohemagglutinin
PMA	Phorbol myristate acetate
PVDF	Polyvinylidene difluoride
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel Electrophoresis
Sf9	*Spodoptera frugiperda* insect cell
S.O.C.	Per 100 ml

Bacto-tryptone	2%
Bacto-yeast extract	0.5%
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$, $MgSO_4$	20 mM
Glucose	20 mM

SSC	Standard saline citrate (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)
SV-40	Simian virus 40
TBS	Tris-buffered saline
Tc	Tetracycline
Tris	Tri-(hydroxymethyl)aminomethane

CHAPTER I

INTRODUCTION

The process of blood cell formation in the bone marrow and lymphoid organs, termed hematopoiesis, is sustained and regulated throughout life by hematopoietic growth factors. These growth factors are known as the colony-stimulating factors (CSFs) and are required for the survival, proliferation and differentiation of granulocyte and macrophage cell lineages (Yunis et al., 1983). Several classes of CSFs can be distinguished by their unique molecular and biological properties. Macrophage-CSF (M-CSF, also known as CSF-1) stimulates the growth of macrophages; granulocyte-macrophage-CSF (GM-CSF) for both granulocytes and macrophages; granulocyte-CSF (G-CSF) for granulocytes; multi-CSF (also known as interleukin-3) stimulates the growth of granulocytes, macrophages, master cells, megakaryocytes and erythroid cells (Metcalf, 1988). Some properties of the four distinct types of CSF in humans are summarized in Table I. One of these factors, human GM-CSF (hGM-CSF), has a broad range of activities. The recombinant hGM-CSF expressed in COS-1 cells stimulates not only neutrophilic, eosinophilic

TABLE I

THE HUMAN HEMATOPOIETIC COLONY-STIMULATING FACTORS

Name	Alternative names	mRNA size (Kb)	Purified from conditioned medium of	Molecular weight[a] (KDa)	Form
GM-CSF	Pluripoietin α, CSF α	1.0	Mo leukemia cells	14-35	Monomer
G-CSF	Pluripoietin, CSF β	2.0	5637 bladder cancer cells	18-22	Monomer
M-CSF	CSF-1	1.8 4.0	Urine, MiaPaCa pancreas cancer cells	45-90	Dimer
Multi-CSF	IL-3	1.0	T-lymphocytes	14-28	Monomer

a. Variations in apparent molecular weight of native CSFs are due to glycosylation differences.

and monocyte-macrophage progenitor cells, but also megakaryocyte colony-forming cells (Emerson et al., 1985). Furthermore, *in vitro* hGM-CSF stimulates mature neutrophils to localize at sites of inflammation (Gasson et al., 1984; Weisbart et al., 1985), enhances their cytotoxicity (Vadas et al., 1983) and stimulates eosinophils to kill parasites (Dessein et al., 1982). *In vivo*, hGM-CSF has been shown to stimulate monkey hematopoiesis (Donahue et al., 1986). Recently, human trials of hGM-CSF in AIDS patients showed that hGM-CSF was well tolerated and that it increased the circulating levels of neutrophils, monocytes and eosinophils (Groopman et al., 1987). hGM-CSF is also being investigated as a form of therapy in cancer, preleukemic conditions and aplastic anemia. Because of the availability of the recombinant CSFs and no significant adverse effect and no toxicity of CSFs trial in animals, the concept, boosting host defense against infection and malignancy, will become the most common applications of CSFs in treating human disease.

The hGM-CSF complete gene (Kaushansky et al., 1986) has been isolated from a human genomic library. It is 2.5 kilobases (Kb) in length, contains four exons and three introns (Miyayake et al., 1985), and is located on chromosome 5q-21 (Huebner et al., 1985). Restriction enzyme analysis of both the Mo-cell line (T-lymphoblast) and human liver DNA

suggests that there is a single gene encoding hGM-CSF (Wong et al., 1985). hGM-CSF cDNA shown in Fig.1 contains a single open reading frame of 432 nucleotides encoding 144 amino acids. hGM-CSF, like other secreted proteins, is synthesized as a precursor from which 17 residues are cleaved to yield a 127 residue mature protein with a molecular mass of 14,459 Da (Wong et al., 1985). The hGM-CSF glycoprotein isolated from the hairy T-cell leukemia line Mo (Gasson et al., 1984; Wong et al., 1985) has a molecular mass of 22 KDa. Recombinant hGM-CSF purified from COS cells has a molecular mass of 18-28 KDa. The variable molecular mass (14-35 KDa.) of hGM-CSF from different cells has been attributed to different glycosylation patterns. GM-CSF has been purified to apparent homogeneity from conditioned medium of murine lung (Burgess et al., 1977) and human T cells (Gasson et al., 1984). Both human and murine GM-CSFs are glycoproteins with apparent molecular weight of 22 and 23 KDa, respectively. The predicted amino acid sequences for both human and murine GM-CSF cDNA clones are 54% identical and nucleotide sequences are 70% conserved (Miyatake et al., 1985). The disulfide structure, which is essential for GM-CSF biological function, is completely conserved between the human and murine growth factors, and the potential carbohydrate modification pattern, two N-glycosylation sites, is similar. Despite these

Fig. 1. DNA sequence and deduced amino acid sequence of hGM-CSF cDNA. The signal peptide cleavage site is indicated by Δ. Two potential N-linked glycosylation sites (Asn-Xaa-Thr/Ser) in the amino acid are marked by ●.

```
                                TCTGGA GG ATG TGG CTG CAG AGC CTG
                                          Met Trp Leu Gln Ser Leu

CTG CTC TTG GGC ACT GTG GCC TGC AGC ATC TCT GCA CCC GCC CGC
Leu Leu Leu Gly Thr Val Ala Cys Ser Ile Ser Ala Pro Ala Arg
                                                  Δ
TGC CCC AGC CCC AGC ACG CAG CCC TGG GAG CAT GTG AAT GCC ATC
Ser Pro Ser Pro Ser Thr Gln Pro Trp Glu His Val Asn Ala Ile

CAG GAG GCC CGG CGT CTC CTG AAC CTG AGT AGA GAC ACT GCT GCT
Gln Glu Ala Arg Arg Leu Leu Asn Leu Ser Arg Asp Thr Ala Ala
                                    •
GAG ATG AAT GAA ACA GTA GAA GTC ATC TCA GAA ATG TTT GAC CTC
Glu Met Asn Glu Thr Val Glu Val Ile Ser Glu Met Phe Asp Leu
          •
CAG GAG CCG ACC TGC CTA CAG ACC CGC CTG GAG CTG TAC AAG CAG
Gln Glu Pro Thr Cys Leu Gln Thr Arg Leu Glu Leu Tyr Lys Gln

GGC CTG CGG GGC AGC CTC ACC AAG CTC AAG GGC CCC TTG ACC ATG
Gly Leu Arg Gly Ser Leu Thr Lys Leu Lys Gly Pro Leu Thr Met

ATG GCC AGC CAC TAC AAG CAG CAC TGC CCT CCA ACC CCG GAA ACT
Met Ala Ser His Thr Lys Gln His Cys Pro Pro Thr Pro Glu Thr

TCC TGT GCA ACC CAG ATT ATC ACC TTT GAA AGT TTC AAA GAG AAC
Ser Cys Ala Thr Gln Ile Ile Thr Phe Glu Ser Phe Lys Glu Asn

CTG AAG GAC TTT CTG CTT GTC ATC CCC TTT GAC TGC TGG GAG CCA
Leu Lys Asp Phe Leu Leu Val Ile Pro Phe Asp Cys Trp Glu Pro

GTC CAG GAG TGA GACCGGCCAGATG AGGCTGGCCA AGCCGGGGAG
Val Gln Glu
CTGCTCTCTC ATGAAACAAG AGCTAGAAAC TCAGGATGGT CATCTTGGAG
GGACCAAGGG GTGGGCCACA GCCATGGTGG GAGTGGCCTG GACCTGCCCT
GGGCACACTG ACCCTGATAC AGGCATGGCA GAAGAATGGG AATATTTTAT
ACTGACAGAA ATCAGTAATA TTTATATATT TATATTTTTA AAATATTTAT
TTATTTATTT ATTTAAGTTC ATATTCCATA TTTATTCAAG ATGTTTTACC
GTAATAATTA TTATTAAAAA TAGCTTCTAA AAAAAAAAA
```

features, human GM-CSF fails to stimulate colony formation in cultures of murine marrow progenitors, and murine GM-CSF fails to stimulate human progenitor or mature blood cells.

Although much effort has been devoted to the purification of the natural human GM-CSF (Wong et al., 1985), G-CSF (Welte et al., 1985), and M-CSF (Wu et al., 1979; Das et al., 1981), they exist in minute amounts from natural sources and the biological characterizations of CSFs are therefore limited. However, a breakthrough in the production of the CSFs has come from the development of molecular cloning techniques. The molecular cloning and sequencing of cDNA of CSFs including hCSF-1 (Kawasaki et al., 1985; Wong et al., 1987), hGM-CSF (Kaushansky et al., 1986; Wong et al., 1985; Lee et al., 1985; Cantrell et al., 1985), hG-CSF (Nagata et al., 1986; Souza et al., 1986), and hmulti-CSF (Yang et al., 1986) have recently been accomplished and the amino acid sequence of those factors have also been deduced from cDNA. hGM-CSF has been produced by recombinant DNA techniques in transfected COS cells (Wong et al., 1985; Lee et al., 1985), yeast (Cantrell et al., 1985; Miyajima et al., 1986) and *E.coli* (Libby et al., 1987). The *E.coli*, yeast, and COS cell systems have some disadvantages. Although hGM-CSF produced in transfected COS cells folds appropriately, it is unfortunately expressed only transiently, and at a relatively

low level of 1 μg of product per milliliter of culture supernatant. Yeast-derived hGM-CSF is produced at somewhat higher levels, but can be derivatized with only yeast-specific carbohydrate moieties. Expression in *E.coli* has other drawbacks, since *E.coli* can neither synthesize glycoproteins nor secrete proteins into the medium. The expression of hGM-CSF in the baculovirus-*Spodoptera frugiperda* (Sf9) cell system provides an alternative which can overcome these problems.

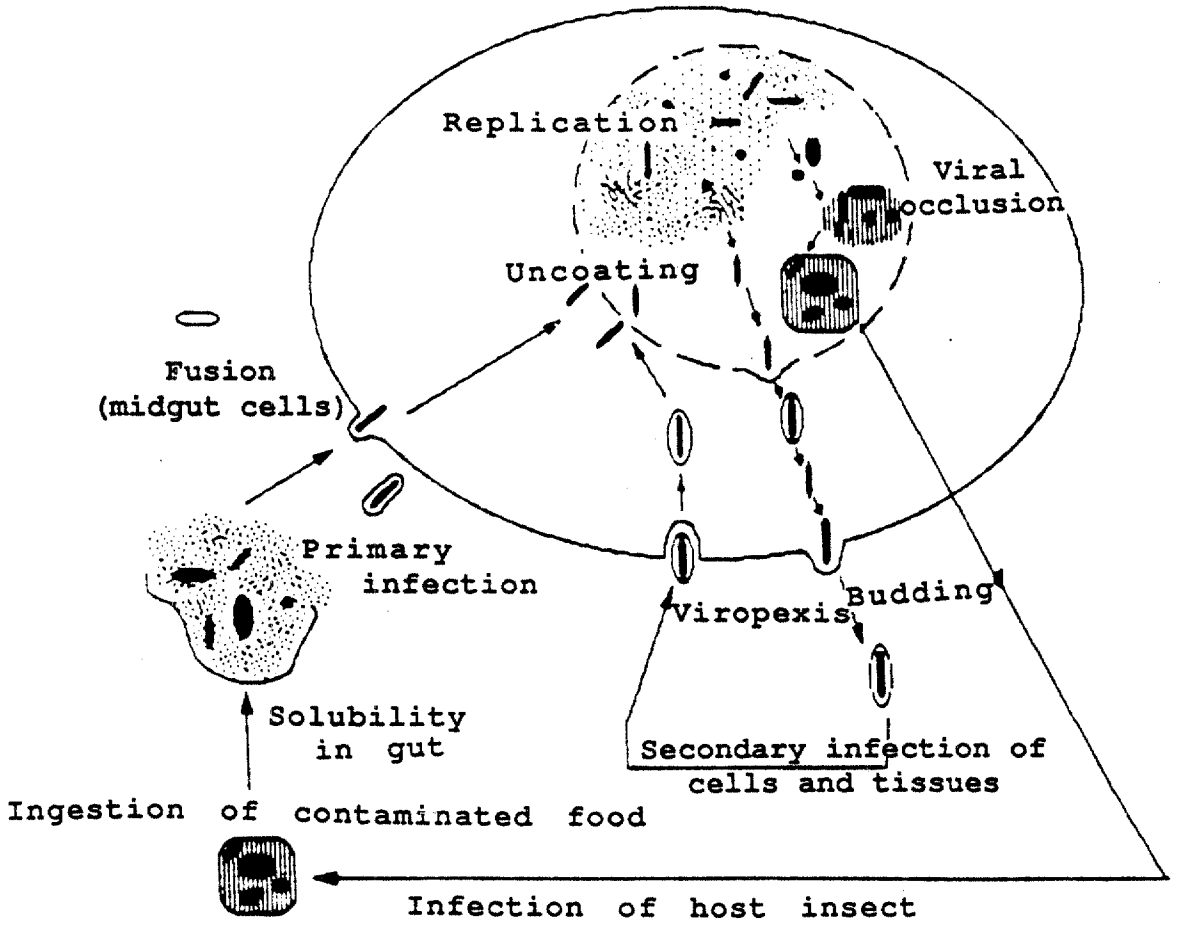
The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) is the prototype virus of the family *Baculoviridae* (Matthews, 1982) and can be used as a host for more than 30 species of Lepidopteran insects (Groner, 1986). AcNPV contains a circular supercoiled double-stranded DNA genome 128 kilobases (Kb) in length (Smith et al., 1978). During AcNPV infection, two forms of viral progeny are produced: extracellular virus particles (ECV), and occluded virus particles (OV) (Volkman et al., 1976). The latter are embedded in proteinaceous viral occlusions, called polyhedra. AcNPV is a very useful helper-independent eucaryotic expression vector for expression of a wide variety of heterologous genes in large amounts (Luckow et al., 1988). Polyhedrin, a late gene product of AcNPV, can account for up to 50% of the total "Coomassie stainable" protein of the cell detected on SDS-polyacrylamide gels

during a lytic infection (Smith et al., 1983). The life cycle of AcNPV in Sf9 cells is summarized in Fig.2. Virus particles enter the cell by fusion or endocytosis, and the viral DNA is uncoated at the nuclear pore or in the nucleus. At about 6 hours post-infection (hpi), the viral DNA begins to replicate. Extracellular virus (ECV) is released from the cell by budding at about 10 hpi. Polyhedrin protein can be detected by 12 hpi, but viral occlusions are not detected until 18-24 hpi. Extracellular virus levels reach a maximum between 36-48 hpi, but the polyhedrin protein continues to accumulate until the infected cells lyse 4-5 days.

Because the polyhedrin gene is nonessential for replication or production of extracellular viruses in cultured cells, vectors that utilize the strong AcNPV polyhedrin promoter to drive the expression of foreign genes have been developed (Smith et al., 1983). By using this approach, large amounts of eucaryotic proteins have been successfully produced, including human fibroblast interferon (Smith et al., 1983), human c-*myc* protein (Miyamoto et al., 1985), and human interleukin 2 (Smith et al., 1985). In addition, insect cells can perform many of the higher eucaryotic posttranslational modifications, including glycosylation (Smith et al., 1983; Kuroda et al., 1986; Estes et al., 1987; Greenfield et al., 1988), extracellular

Fig.2. Baculovirus life cycle. The schematic depicts the unique biphasic life cycle of a typical baculovirus. In the environment a susceptible insect ingests the viral occlusions from a food source. The crystal dissociates in the gut of the susceptible insect to release the infectious virus particles which invade the gut cells, penetrate to the nucleus and uncoat. Viral DNA replication is detected by 6 hours. By 10-12 hours post infection extracellular virus bud from the surface to infect other cells and tissues. Late in infection (18-24 hours post infection) the polyhedrin protein assembles in the nucleus of the infected cell and virus particles become embedded in the proteinaceous occlusions. The viral occlusions accumulate to large numbers and the cell lyses. The viral occlusions are responsible for horizontal transmission among susceptible insects, the extracellular virus is responsible for secondary and cell to cell infection in cultured cells or the insect host (Summers et al., 1988, slightly modified).

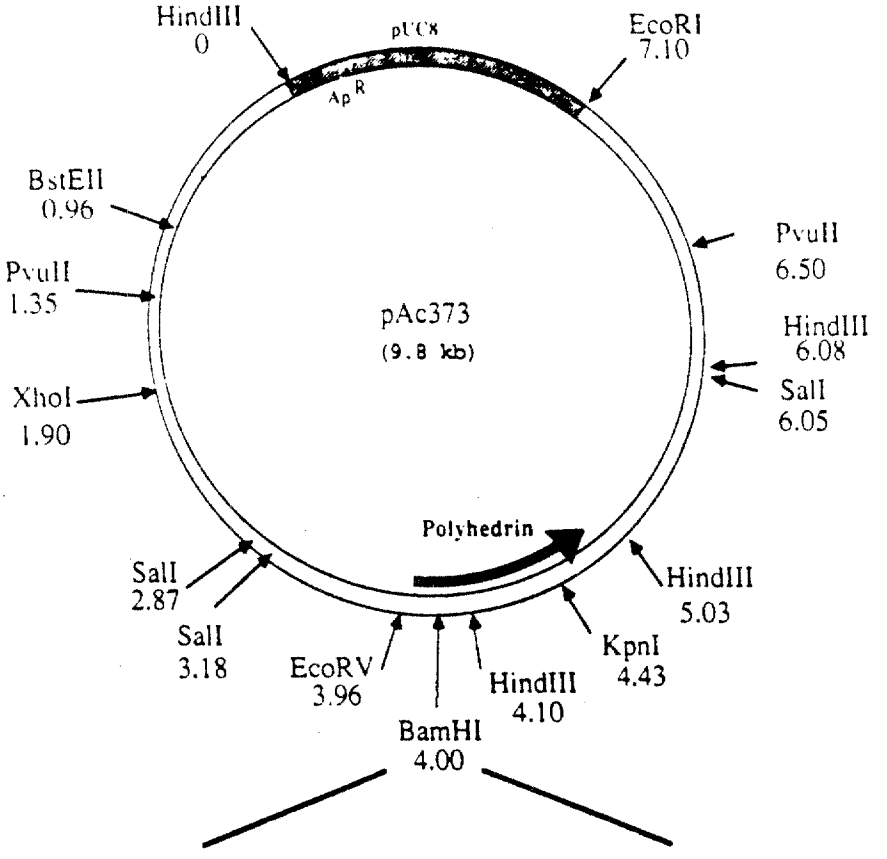
Baculovirus Life Cycle



secretion (Smith et al., 1983; Smith et al., 1985; Maeda et al., 1985), accurate signal peptide cleavage (Smith et al., 1983; Smith et al., 1985; Matsuura et al., 1986; Possee et al., 1986; Madisen et al., 1987; Kuroda et al., 1986; Maeda et al., 1985), nuclear translocation (Miyamoto et al., 1985; Jeang et al., 1987; Ollo et al., 1987) and phosphorylation (Miyamoto et al., 1985; Jeang et al., 1987; Ollo et al., 1987). These features of the baculovirus system make it an attractive proposition for the large-scale production of recombinant hGM-CSF.

Because of the large size of the baculovirus genome, most recombinant virus construction relies on *in vivo* recombination or replacement of a viral allele with a gene of interest (Smith et al., 1983; Maeda et al., 1985; Miller, 1981; Miller et al., 1983; Pennock et al., 1984). Most transfer vectors contain sequences from AcNPV including the promoter of the polyhedrin gene and varying amounts of 5' and 3' viral DNA flanking the polyhedrin gene cloned in a high copy number bacterial plasmid. A variety of transfer vectors suitable for production of fused or nonfused proteins have been constructed. Currently, the most widely used transfer vector for introducing foreign genes into AcNPV is pAc373 (Smith et al., 1985), shown in Fig.3. pAc373, used for production of nonfused proteins, was derived from a plasmid

Fig. 3. AcNPV transfer vector pAc373. The circular restriction map is of the AcNPV transfer vector pAc373. Numbers at restriction sites indicate distance in kilobase pairs (kbp) from the *Hind*III end of the pUC8 backbone; the thick arrow marks the polyhedrin sequence; Ap^r, ampicillin resistance gene. A unique *Bam*HI site is located following position -8 with respect to the polyhedrin initiator codon, ATG, where the A is nucleotide +1. The transfer vector pAc373 has a deletion between -9 and +175 (Summers et al., 1988, slightly modified).



-16 -8 +177

5'-AAAAAAACC CGAGATCCGCGGATCC TTTCCT

BamHI

No sites for: SmaI, PstI, BglII, XbaI, SstI

comprised of a 7 Kb *Eco*RI fragment containing the AcNPV polyhedrin gene cloned into the *Eco*RI-*Hind*III fragment of pUC8. The foreign gene sequence in the recombinant plasmid can be transferred to wild-type AcNPV by homologous recombination with a cell transfected with both the plasmid and wild-type virus.

The major objective of this research is to express the hGM-CSF gene at high levels using the baculovirus vector system and to produce the biological activity of recombinant hGM-CSF in insect cells. To achieve this purpose, the specific aims included: (1) isolation of the hGM-CSF fragment from p91023(B), (2) cloning the hGM-CSF gene into AcNPV transfer vector, (3) transferring the hGM-CSF gene into the AcNPV genome, (4) identification and characterization of recombinant hGM-CSF. In this study, this expression system was used in the production of hGM-CSF to study the protein processing of the hGM-CSF protein in insect cells. Under the control of the strong polyhedrin promoter in the recombinant virus, abundant quantities of active hGM-CSF protein is secreted into the culture medium.

CHAPTER II

PURIFICATION AND IDENTIFICATION OF hGM-CSF PLASMID

Complementary DNA (cDNA) clones encoding hGM-CSF have been reported by Wong et al (Wong et al., 1985) from a human T-cell leukemia virus (HTLV)-transformed T-lymphoblast cell line Mo stimulated with phytohemagglutinin (PHA) and phorbol myristate acetate (PMA). If the hGM-CSF cDNA is inserted in p91023(B) DNA then, the hGM-CSF gene will be transferred from p91023(B) DNA to a baculovirus expression vector (pAc373). The first step is to isolate p91023(B) DNA from bacteria and to confirm the insertion of the hGM-CSF cDNA by nucleic acid hybridization. p91023(B) is subsequently transfected into COS cells to confirm the production of the functional hGM-CSF protein.

Materials and Methods

The Bacto tryptone, Bacto yeast extract and Bacto agar for bacterial culture were obtained from Difco, Detroit, MI. Tetracycline, lysozyme and DEAE-dextran were purchased from Sigma, ST. Louis, MO. Ribonuclease (Bovine Pancreas) and

Sephacryl S-300 Superfine were from Pharmacia, Piscataway, NJ. Hybond-N membrane was obtained from Amersham, Arlington Heights, IL. X-Omat AR film and film holders were purchased from Kodak, Rochester, NY. Intensifying screens (Cronex Quanta III) were from Du Pont, Wilmington, DE. The plasticware for tissue culture was purchased from Falcon Products, Los Angeles, CA. Dulbecco's modified Eagle's (DME) medium, fetal calf serum, horse serum, new-born calf serum, penicillin and streptomycin were purchased from GIBCO Grand Island, NY. Sephracell-MN was from Sephratech, Oklahoma City, OK.

Extraction and Purification of Plasmid DNA (GM-CSF) from *E.coli*: A single colony carrying hGM-CSF plasmid (p91023 (B); ATCC, 39754) was isolated from a freshly streaked plate, inoculated in 5 ml LB medium (1% w/v Bacto tryptone, 0.5% Bacto yeast extract and 1% NaCl pH 7.5) containing 12.5 µg/ml tetracycline and grown overnight on a shaker at 37°C. The next day, 5 ml of overnight culture was used to inoculate 500 ml LB medium with tetracycline and grown at 37 °C overnight. The cells were spun down by centrifugation for 10 min at 5000 rpm in a Beckman JA-10 rotor. The supernatant was discarded and the pellet was resuspended in 20 ml resuspenion buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.8, and 1

mM EDTA). The cell solution was distributed into four 50 ml centrifuged tubes and centrifuged for 10 min at 5000 rpm in a Beckman JA-20 rotor. The bacterial pellet was resuspended in 3 ml solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing 5 mg/ml lysozyme, and incubated at room temperature for 10 min. Nine ml solution II (0.2 M NaOH, 1% SDS) was then added and the mixture allowed to stand for 10 min on ice. Four and half ml of Solution III (5 M potassium acetate pH 4.8) was added and the total mixture allowed to stand on ice for additional 10 min. The solution was centrifuged at 15000 rpm for 30 min at 4^{o}C, and the supernatant was transferred to a new centrifuge tube. The DNA was precipitated by adding 2 volumes (vol) of ethanol and standing for 0.5-1 hr at -70^{o}C. The precipitated DNA was centrifuged at 15000 rpm for 20 min and the alcohol was removed. The pellet was dissolved in 5 ml TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA). The solution was then treated with ribonuclease (heat-treated at 100^{o}C for 10 min in 10 mM Tris-HCl, pH 7.5, and 15 mM NaCl to inactive deoxyribonuclease) at a concentration of 20-50 µg/ml for 30 min at 37^{o}C. The solution was extracted with an equal vol of phenol, chloroform and iso-amyl alcohol (25:24:1) and centrifuged at 10000 rpm for 10 min. The aqueous phase was transferred to a new tube and 0.1 vol of 3 M Na acetate (pH

5.2) and 2 vol ethanol were added. The mixture was incubated for 30 min at -70°C and then centrifuged at 15000 rpm for 30 min at 4°C. The pellet was dissolved in 0.5 ml TE buffer and applied to a gel filtration column as described below. The column (1.5 x 50 cm) was packed with Sephacryl S-300 Superfine. This was equilibrated at room temperature with 10 mM Tris-HCl pH 7.6, 0.2 M NaCl and 0.5 mM EDTA. The DNA preparation from a culture prepared as described in the preceding section was applied to the column, and then eluted at room temperature using column buffer at a flow rate of 7 ml/hr and collecting 1.2 ml fractions. The elution was monitored by UV spectrophotometry at 260 nm. The fractions of plasmid DNA were pooled and concentrated by ethanol precipitation as described previously.

Identifying Plasmid DNA (hGM-CSF) by Dot Hybridization: two-three µl aliquots of DNA (20 ng) were spotted on a fresh Hybond-N (Nylon) membrane. The filters were marked to ensure correct orientation. With the spotted side facing up, the filters were first laid for 1 min on filter paper saturated in 0.5 M NaOH and 1.5 M NaCl denaturing solution and subsequently treated similarly with 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2 neutralizing solution for a further 1 min. The filters were air-dried at room temperature and irradiated for

2-5 min under UV light. Filters were processed in heat-sealed polyester bags. Prehybridization conditions were as follows: 5X SSC, 15X Denhardt's solution and 500 µg/ml salmon sperm DNA for 2-4 hr at 50°C with shaking. A 5'-end hGM-CSF probe (3'-ACC GAC GTC TCG GAC GAC GAG-5') or a 3'-end hGM-CSF probe (3'-ACG ACC CTC GGT CAG GTC CTC-5') was labeled in 50 µl kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT) and T4 polynucleotide kinase (10 U) in the presence of 100 µCi [γ-^{32}P] ATP (4082 Ci/mmole) at 37°C for 1 hr. One-tenth of the labeled probe (10 µCi) was added to prehybridization solution and the filter was hybridized at 50°C overnight. The volumes used for prehybridization and hybridization were around 5-10 ml in a heat-sealed bag. Washing conditions used were 1X SSC and 0.1% SDS at 50°C for two washings. The filters were air-dried and exposed to X-Omat AR film (Kodak) overnight at -70°C using an intensifying screens.

Transient Expression of hGM-CSF in Mammalian Cells: M6 COS-1 monkey cells (Gluzman, 1981) were grown routinely in Dulbecco's modified Eagle's medium (DME) containing 10% heat-inactivated (30 min at 50°C) fetal calf serum (FCS). Twenty-four hr prior to transfection, the cells were split 1:6 into fresh plates. A 4 µg sample of DNA was used to transfect a 60 mm dish of COS-1 cells by DEAE-dextran-mediated DNA

transfection. The 4 μg of DNA was dissolved in 20 μl TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$). The media was removed from the plates which were then washed with 3 ml of 1X PBS. Then 4 ml of 4% NuSerum in DME was added to each plate. Twenty μl of resuspended DNA was added to 60 μl warm (30°C to 37°C) 10 mg/ml DEAE-dextran in TBS. The DNA/DEAE-dextran was added drop by drop to each plate. The DNA/DEAE-dextran/NuSerum/ was removed from plates after 4 hr incubation in a tissue culture incubator. The cells were shocked in 1.5 ml of 10% DMSO in PBS for 2 min at room temperature. After 2 min, this was replaced with 3 ml DME containing 4% FCS. The transfection COS-cell-conditioned medium was harvested 72 hr after DNA transfection and activity was measured in human cord blood CSF assay (Ogawa et al., 1983; Knudtzou, 1984).

Biological Assay for hGM-CSF: The soft agar assay of human mononuclear cells from human cord blood was carried out as described below. The mononuclear cells were isolated by density centrifugation on Sepracell-MN and cultured at a concentration of 2×10^5 cells per ml of the assay medium after the adherent cells were depleted by 2-3 hr incubation in tissue culture dishes. The culture medium components were double strength DME medium containing 20% fetal calf serum,

20% horse serum, 19.8 µg/ml asparagine, 75 µg/ml DEAE Dextran, 100 Units/ml penicillin, 100 µg/ml streptomycin and 7.6 mg/ml sodium bicarbonate and mixed with equal vol of 0.6% agar at 37°C. Approximately 2 x 10^5 nonadherent mononuclear cells from cord blood were dispensed into each 35 mm petri dishe containing 0.2 ml of sample to be assayed. The dishes were swirled to ensure an even distribution and left at room temperature for 30 min, and then incubated for 10-12 days at 37°C under CO_2. Using a dissecting microscope aggregates of 50 or more cells were scored as one colony. One unit of CSF activity is defined as the amount of activity that stimulates the formation of one colony under the specified conditions of the assay.

Results

It was necessary to demonstrate that the p91023(B) plasmid DNA actually harbors the GM-CSF gene sequence for further expression of hGM-CSF in COS and insect cells. The p91023(B) plasmid DNA was isolated from bacteria and hybridized with a 21-mer DNA probe. Fig.4 shows the intensity of each dot correlated directly with the probe-specific plasmid DNA concentrations in p91023(B) samples. The plasmid DNA, which gave a very intense hybridization

Fig. 4. Dot blot hybridization of p91023(B) plasmid DNA. Plasmid DNA was prepared by the alkaline lysis method (Birnboim et al., 1979; Ish-Horowicz et al., 1981). The samples of plasmid DNA were spotted on a nylon filter and divided in two. One was hybridized to a probe (3'-ACC GAC GTC TCG GAC GAC GAG-5') derived from the 5'-region of the hGM-CSF cDNA clone (lanes 1,2) and the other was hybridized to another probe (3'-ACG ACC CTC GGT CAG GTC CTC-5') derived from the 3'-region of the hGM-CSF cDNA clone (lanes 3,4). Lanes 1 and 3 were the plasmid DNA of p91023(B) (20 ng of serial 1:5 dilution). Lane 2 and 4 were pBR322 plasmid DNA (20 ng of serial 1:5 dilution).

1

2

3

4

signal, was digested with *Eco*RI. The hGM-CSF cDNA was approximately 800 bp in size as shown in Fig.5, which was the same size as described previously (Wong et al., 1985). Furthermore, the transient expression vector carrying GM-CSF cDNA was transfected into COS-1 cells to confirm that the clone could express a functional protein in mammalian cells. Results of human cord blood assays showed that biologically active hGM-CSF was produced by transfected COS cells (Table II). hGM-CSF was produced in transfected COS cells at level of 1.2×10^3 U/ml (1.5×10^5 cells/ml).

Discussion

The human cDNA segment harbored in p91023(B) contains the full-length hGM-CSF gene, as indicated from dot blot hybridization and restriction endonuclease analysis done in this study. Wong et al (Wong et al., 1985) described the hGM-CSF cDNA which contains a single open reading frame of 432 nucleotides encoding 144 amino acids. hGM-CSF, like other secreted proteins, is synthesized as a precursor that is cleaved after residue 17 to yield a 127 residues mature protein. The hGM-CSF cDNA in the p91023(B) expression vector provided another assay to confirm that the full-length cDNA clone was expressed in mammalian cells. COS-1 monkey cells

Fig. 5. *Eco*RI cleavage of p91023(B) plasmid DNA. Plasmid DNA was digested with *Eco*RI, electrophoresed on a 0.7% agarose gel, transferred to a nylon filter and hybridized to ^{32}P-labeled hGM-CSF probe (5'-end hGM-CSF). Lane 1 was *Hind*III-digested λ DNA. Lane 2 was *Eco*RI-digested p91023(B) DNA.(A) Ethidium bromide-stained gel (B) Autoradiogram of the nylon filter prepared from gel shown in (A).

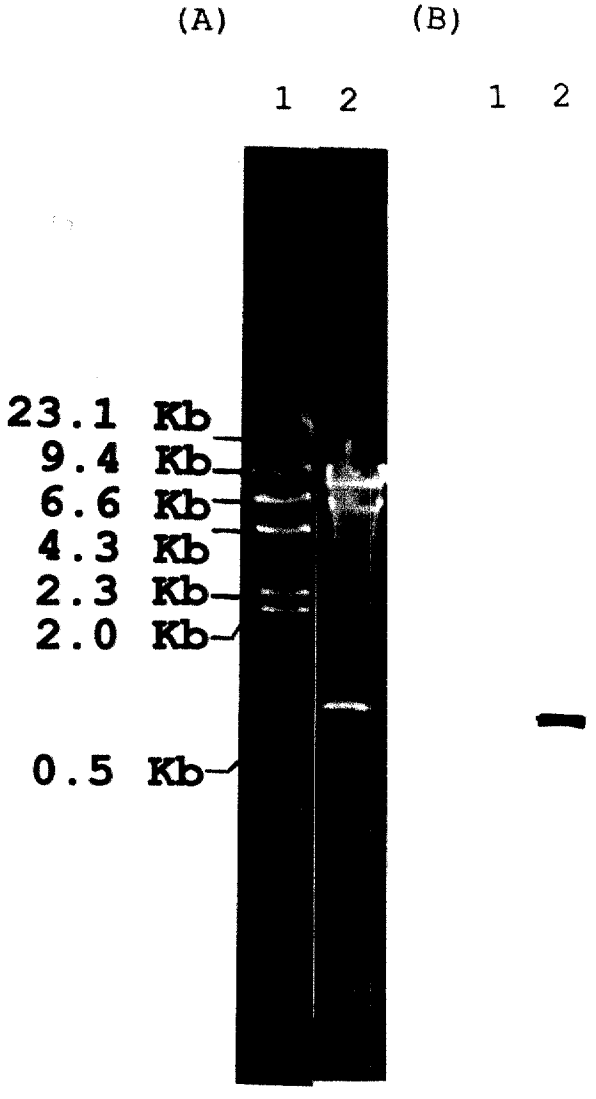

(A)
(B)
1
2
1
2
23.1 Kb
9.4 Kb
6.6 Kb
4.3 Kb
2.3 Kb
2.0 Kb
0.5 Kb

TABLE II

HGM-CSF ACTIVITY IN SUPERNATANTS FROM COS CELLS TRNSFECTED WITH PLASMID DNA

Supernatants	CSF Activity (U/ml)
p91023(B)	1290
pBR322	15
H_2O	10

* All the data are from the average of duplicate samples and the deviation is less than 10%.

were used as the recipient for this plasmid because the SV40 large T antigen they produce drives the replication of the p91023(B) DNA from the SV40 origin of replication. The amplified copy number of the plasmid in COS-1 cells increases the transient expression of genes on the plasmid. The production of biologically active hGM-CSF after transfection of COS-1 cells with p91023(B) and dot blot hybridization with N-terminal and C-terminal hGM-CSF sequence probes provides evidence that the cDNA clone encodes hGM-CSF gene.

CHAPTER III

CLONING hGM-CSF GENE INTO AcNPV TRANSFER VECTOR

The transfer vector of baculovirus (Fig.3) contains sequences from AcNPV, including the polyhedrin promoter and 5' and 3' viral DNA flanking the polyhedrin gene cloned into a high copy number bacterial plasmid. The hGM-CSF sequence cloned into the plasmid p91023(B) was inserted into the transfer vector pAc373. The segment of hGM-CSF cDNA was removed from p91023(B) by first digesting with *Eco*RI, filling in with Klenow fragment, ligating with *Bam*HI linker, and then digesting with *Bam*HI. This segment was transferred into the baculovirus polyhedrin plasmid pAc373 which had been digested with *Bam*HI and treated with bacterial alkaline phosphatase. A new construct (pAc373GM-CSF) was obtained, containing the intact coding sequence for hGM-CSF in the proper orientation downstream of the strong polyhedrin promoter.

Materials and Methods

The transfer vector of pAc373 was kindly provided by Dr. Max D. Summers (Texas A & M University, College Station).

Restriction enzymes and T4 DNA ligase were purchased from B.R.L., Gaithersburg, MD. Klenow fragment and T4 polynucleotide kinase were obtained from U.S.B., Cleveland, OH. The linker of *Bam*HI and 21-base oligonucleotide were generously provided by O.C.S., Sanger, TX.

Recovery of hGM-CSF Fragment from Agarose Gel: After restriction endonuclease (*Eco*RI) digestion of the DNA, fragments were separated by electrophoresis on a 0.7% agarose gel (9 x 5.5 cm). Gels were run in TBE buffer (50 mM Tris-HCl pH 7.6, 50 mM boric acid, 2.5 mM Na_2EDTA) at 100 volts for 1 hr. The DNA fragments in the gel were stained by soaking in ethidium bromide (0.5 μg/ml), and bands were illuminated with a long-wave UV light source for visualization. Troughs were made in front of bands with a sharp scalpel and filled with TBE buffer. The electrophoresis was resumed and the fluid was recovered from the trough every 2 or 3 min. The eluate was extracted twice with phenol, once with phenol/chloroform and once with chloroform. The upper phase was carefully transferred with a pipette to a clean microfuge tube. To precipitate the DNA fragment, 0.5 vol of 3 M sodium acetate (pH 5.2) was added, followed by 2 vol of cold ethanol. The tube was then placed at -70°C for at least 10 min and centrifuged for 15 min at

4°C to collect the precipitate. The ethanol was decanted and the precipitate was washed once by adding 1 ml of cold ethanol, and the tube was recentrifuged for 15 min. The ethanol was decanted and the pellet was dried under vacuum.

Fill-In of the Recessed 3'-Ends of DNA: The DNA fragment was resuspended in 0.1X TE buffer for filling in the 3'-recessed restriction site by the large fragment of DNA pol I (Klenow fragment). The 30 µl fill-in reaction containing 1 µg of hGM-CSF fragment and 0.5 units of Klenow enzyme in buffer (7 mM Tris-HCl pH 7.4, 7 mM $MgCl_2$, 1 mM DTT, 10 mM NaCl, 16 µM dNTP) was incubated for 10-15 min at room temperature. The reaction mixture was stopped by adding 2 µl of 0.25 M EDTA. The blunt-end hGM-CSF fragment was purified by phenol extraction and ethanol precipitation.

Phosphorylation of *Bam*HI Linker (5'-CCGGATCCGG-3'): Two µg (5 µl) of oligodeoxynucleotide linkers were mixed in kinase buffer with T4 polynucleotide kinase (60 units) at 37°C for 1 hr (total volume of 75 µl).

Ligation of Linker to Blunt-End hGM-CSF Fragment: The ligation reaction with T4 DNA ligase was carried out under the following conditions: 1 µg of blunt-end hGM-CSF fragment

(3 pmole), 500 ng phosphorylated linker (150 pmole ends), 3.5 µl of 10X ligation buffer (660 mM Tris-HCl pH 7.6, 66 mM $MgCl_2$, 100 mM DTT, 4 mM ATP) and 5 units of T4 DNA ligase were incubated for 24 hr at 4°C.

Removal of Excess Linker after Ligation: After ligation of the linker to the hGM-CSF fragment for 24 hr, the reaction mixture was incubated at 65°C for 10 min. The ligation reaction mixture was digested with *Bam*HI restriction enzyme by adding the following components: 10 µl of 10X restriction enzyme buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl), 3 µl of *Bam*HI restriction enzyme (30 units/3 µl) and 52 µl of ddH_2O. The digestion reaction was incubated at 37°C overnight and the excess linker was removed by filtration with Centricon-30 microconcentrator (Amicon). The concentrate (hGM-CSF fragment) was collected after centrifugation in a fixed angle rotor at 6000 rpm. The hGM-CSF fragment (450 ng/6.5 µl) was used for integration into plasmid pAc373 (150 ng/1.5 µl) that has been made linear with *Bam*HI and treated with bacterial alkaline phosphatase. The fragments were ligated with sticky ends in 1 µl of 10X ligase buffer and 1 µl of T4 DNA ligase (1 U/µl) at 14°C overnight. After transformation of $CaCl_2$-treated *Escherichia coli* JM101 cells, recombinant colonies were screened by hybridization

with 5'-end ^{32}P labeled 21-mer oligonucleotide.

Transformation of *E.coli* JM101 by the Calcium Chloride: The *E.coli* JM101 culture was grown at 37°C and treated with $CaCl_2$ as described by Maniatis (Maniatis et al., 1982). One hundred µl competent cells were transferred to prechilled 5 ml test tubes and 5-10 µl of DNA was added. The tubes were placed on ice for 30 min. The transformation mixture was placed at 42°C for 2 min and chilled in an ice bath for 2 min. Then, 900 µl of room temperature S.O.C. broth (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgCl_2$, $MgSO_4$ and 20 mM glucose) was added to the transformation mixture. The transformed cells were incubated at 37°C for 1 hr with shaking around 200 rpm and plated on LB plates with 50 µg/ml ampicillin.

Identification of Recombinant pAc373GM-CSF: Bacterial transformants were grown on selective ampicillin plates until colonies were easily visible (0.5-1 mm). Fresh Hybond-N filters were laid on plates and allowed to bind colonies for 1 min. The filters and plates were marked using a pen to ensure correct orientation of colonies. With the colonies facing up, the filters were first laid for 7 min on filter paper saturated in 0.5 M NaOH and 1.5 M NaCl denaturing solution and subsequently treated similarly with 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2 neutralizing solution for 3 min. The

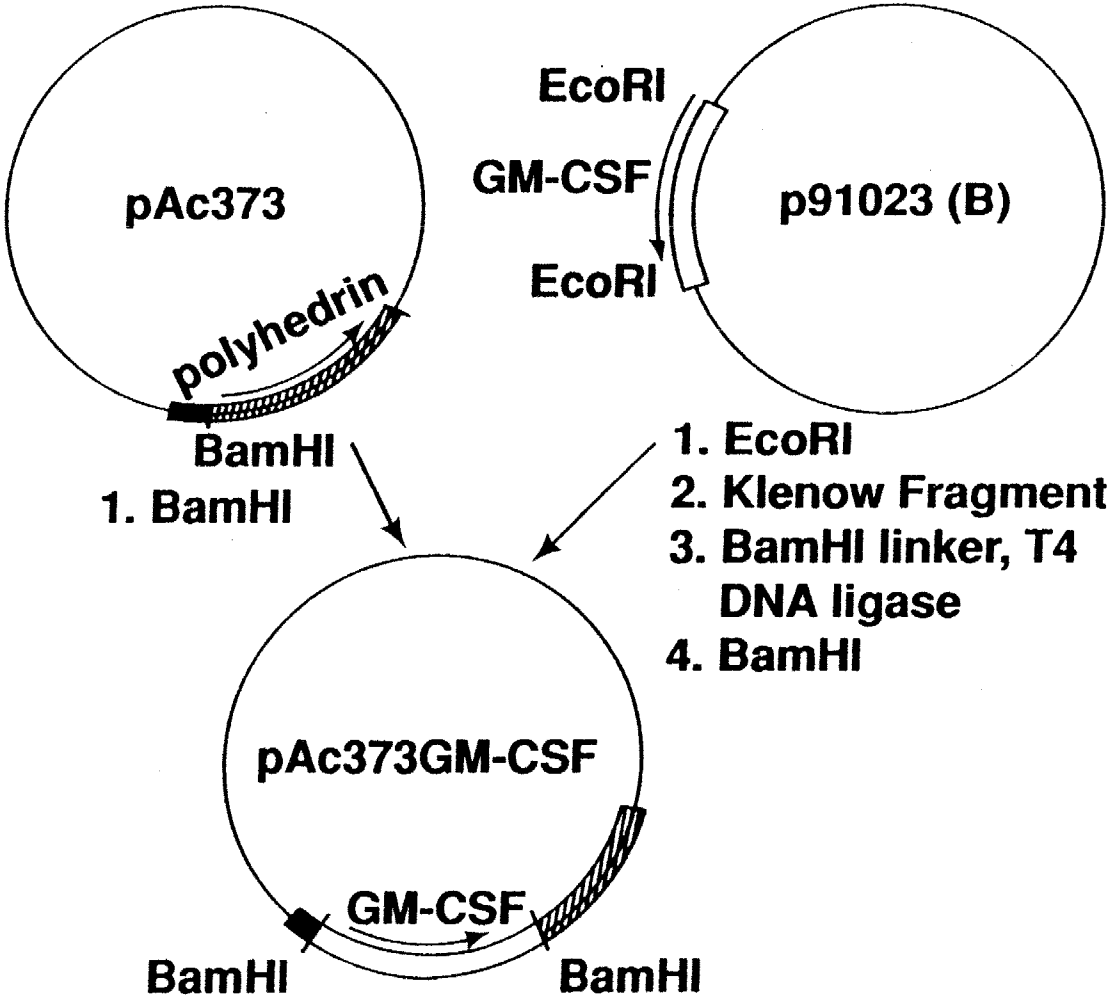
filters were washed by vacuum filtration with 20 ml chloroform and air-dried at room temperature. The filters were irradiated for 2-5 min under UV light. Filters of prehybridization and hybridization were processed in heat-sealed polyester bags as described earlier.

Determination of Orientation of Inserted DNA: The positive clones were picked up and grown in 3 ml LB medium after colony hybridization. Plasmid DNA was prepared by the alkaline lysis method (Birnboim et al., 1979; Ish-Horowicz et al., 1981). Miniprep DNA was double digestion with *Eco*RV and *Pst*I and run on a 0.7% agarose gel in TEB buffer. The gel was stained with ethidium bromide and photographed, soaked once in 0.25 M HCl for 10 min, and once in 1.5 M NaCl and 0.5 M NaOH for 30 min. The DNA was transferred to a nylon filter by blotting in 0.25 M NaOH/1.5 M NaCl for 8-20 hr. The filter was processed and hybridized with 3'-end ^{32}P hGM-CSF probe as described earlier.

Results

As shown in Fig.6, a segment of hGM-CSF DNA from the plasmid p91023(B) was inserted into the pAc373 transfer vector. These recombinant molecules were then inserted into

Fig. 6. Construction of recombinant transfer vector pAc373GM-CSF. The *Eco*RI fragment of hGM-CSF was filled in with Klenow Fragment. *Bam*HI linkers were added with T4 DNA ligase. The fragment was treated with *Bam*HI and inserted into the unique *Bam*HI site of the transfer vector. The resultant plasmid, pAc373GM-CSF, contained the hGM-CSF sequence placed downstream of the strong polyhedrin promoter.


pAc373
polyhedrin
BamHI
1. BamHI
EcoRI
GM-CSF
EcoRI
p91023 (B)
1. EcoRI
2. Klenow Fragment
3. BamHI linker, T4
DNA ligase
4. BamHI
pAc373GM-CSF
GM-CSF
BamHI
BamHI

competent JM101 cells by transformation. Approximately 5-10% of transformants were found to contain the hGM-CSF insert by colony hybridization as shown in Fig.7. The positive colonies were picked up and the recombinant plasmid DNA was prepared by the alkaline lysis method. In Fig.8, the inserted hGM-CSF was around 0.8 Kb on a 1% agarose gel when the recombinant plasmid DNA was digested with *Bam*HI. There are two possible orientations for the insertion of the *Bam*HI fragment. These can be distinguished by the location of an asymmetrically placed *Eco*RV site relative to the *Pst*I site. The orientation of the insert in pAc373 was determined by double digestion with *Eco*RV and *Pst*I followed by Southern blotting (Southern, 1975). In Fig.9A, fragments in size of 10.5 kb and 0.16 kb were produced by double digestion in which the hGM-CSF sequence was inserted in the proper orientation of the pAc373 plasmid DNA. The 0.16 Kb fragment was too small to detect on a 0.7% agarose gel. In Fig.9B, the recombinant DNA was hybridized with an ^{32}P labeled 21-mer hGM-CSF probe to confirm that the recombinant DNA carried the inserted hGM-CSF fragment. As shown in Figs.9A and 9B (lane 7), the 0.9 Kb fragment detected in agarose gel electrophoresis and Southern blot analysis was inserted in the wrong orientation of pAc373.

Fig. 7. Screening of pAc373GM-CSF. Autoradiogram of a Nylon filter replica prepared from a 100 mm plate containing 50 recombinant bacterial colonies. The colonies were lysed and hybridized to a 21-mer hGM-CSF probe. The arrows indicate the position of positive colonies.

Fig. 8. Restriction endonuclease analyses of pAc373GM-CSF. Plasmid DNA prepared from positive colonies was digested with *Bam*HI and electrophoresed on a 0.7% agarose gel. The presence of around 800 bp fragments was indication of an inserted hGM-CSF gene. Lane 1 was *Hind*III-digested λ DNA. Lanes 2-8 were *Bam*HI-digested plasmid DNA from positive colonies.

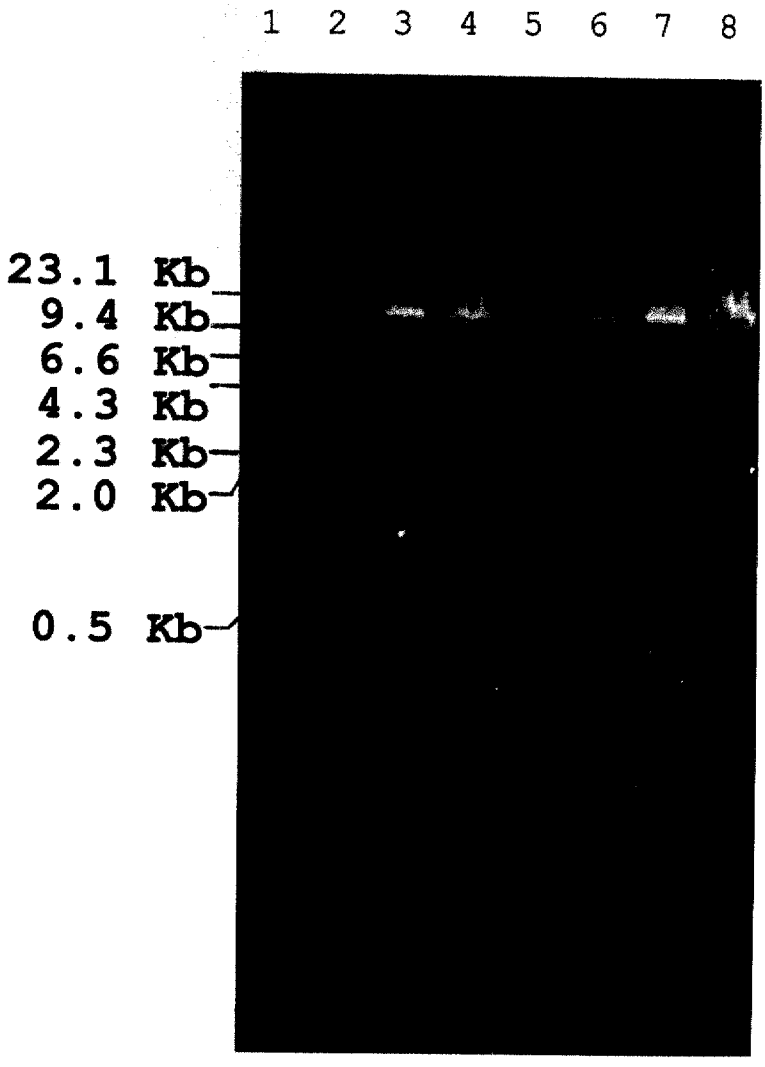
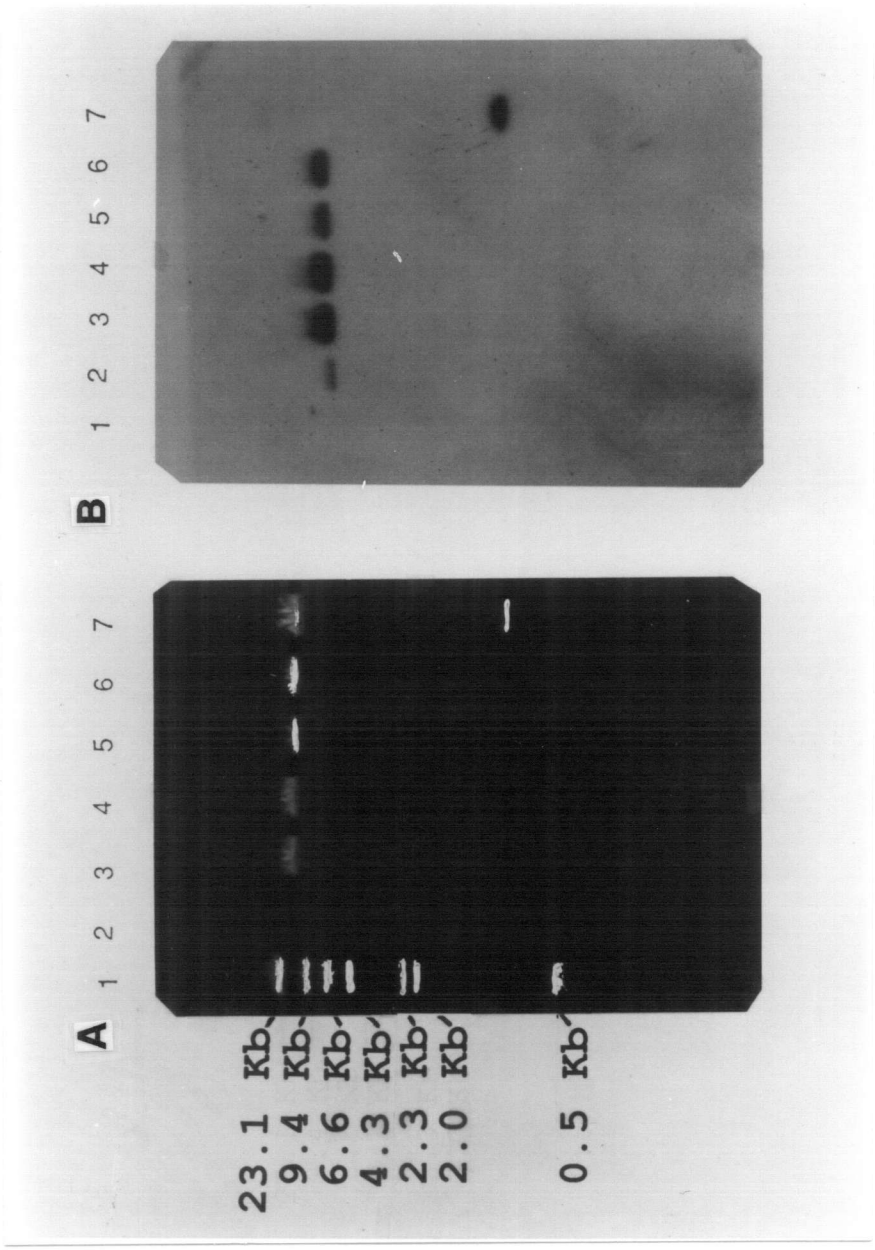

1 2 3 4 5 6 7 8
23.1 Kb
9.4 Kb
6.6 Kb
4.3 Kb
2.3 Kb
2.0 Kb
0.5 Kb

Fig. 9. *Eco*RV and *Pst*I double digestion of plasmid DNA from pAc373GM-CSF. Plasmid DNA from positive colonies were digested with *Eco*RV and *Pst*I, fractionated on a 0.7% agarose gel, transferred to a nylon filter and hybridized to ^{32}P-labeled hGM-CSF probe (3'-end hGM-CSF). Lane 1 was *Hind*III-digested λ DNA. Lanes 2-7 were *Eco*RV- and *Pst*I-digested plasmid DNA from positive colonies (A) Ethidium bromide-stained gel (B) Autoradiogram of the nylon filter prepared from gel shown in (A).


A
1 2 3 4 5 6 7
23.1 Kb
9.4 Kb
6.6 Kb
4.3 Kb
2.3 Kb
2.0 Kb
0.5 Kb
B
1 2 3 4 5 6 7

Discussion

The transfer vector pAc373 has a unique *Bam*HI site following the polyhedrin promoter. *Bam*HI sites were created on the end of the hGM-CSF fragment and the *Bam*HI sticky ended hGM-CSF was then inserted into pAc373. Approximately 5-10% of transformants were found to contain the inserted hGM-CSF fragment indicating successful attachment of the *Bam*HI linker. The orientation of the insert in pAc373 was determined by double digestion with *Eco*RV, which exists in the polyhedrin promoter, and *Pst*I, which is located in the 5' leader sequence of the hGM-CSF gene. Double digestion of the insert in the proper orientation is expected to produce fragments of size 0.16 Kb and 10.5 kb, while the wrong orientation would result in 0.9 kb and 9.7 kb fragments. To confirm the orientation of gene inserts into the transfer vector, the DNA from transformants was treated with *Eco*RV and *Pst*I and then electrophoresed on a 0.7% agarose gel and hybridized with a 3' end hGM-CSF probe using the Southern blot method.

CHAPTER IV

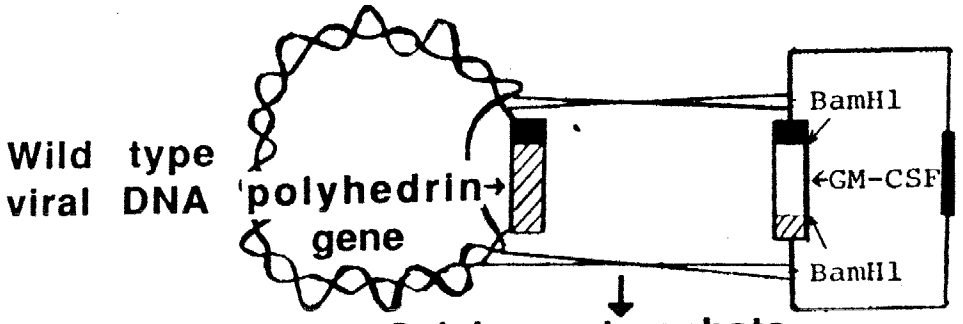
TRANSFERRING hGM-CSF GENE INTO THE AcNPV GENOME

Recombinant plasmids containing inserts in the correct transcription orientation were designated pAc373GM-CSF. As shown in Fig.10, transfer of the hGM-CSF gene from these plasmid vectors into the AcNPV genome was achieved by cotransfection, using the calcium phosphate precipitation method. *In vivo* recombination events yielded a small population of recombinant viruses with hGM-CSF sequences incorporated into the original viral genome. At three or four days post-transfection, the medium had a virus titer of about 10^7 plaque forming units (pfu/ml). The recombinant viruses were identified and purified by plaque hybridization.

Materials and Methods

Spodoptera frugiperda (Sf9) was obtained from ATCC, Rockville, MD. Yeastolate and Lactalbumin Hydrolysate were purchased from Difco, Detroit, MI. Grace's medium was from GIBCO, Grand Island, NY.

Fig. 10. Schematic procedure for cloning hGM-CSF gene and screening recombinant plaques. An hGM-CSF fragment is inserted into a transfer vector in the proper orientation downstream of the polyhedrin promoter. Sf9 insect cells are transfected with a mixture of viral and plasmid DNAs and the viral progeny are plated at 100-1000 plaques per plate. Recombinant viruses are identified visually or by DNA hybridization.



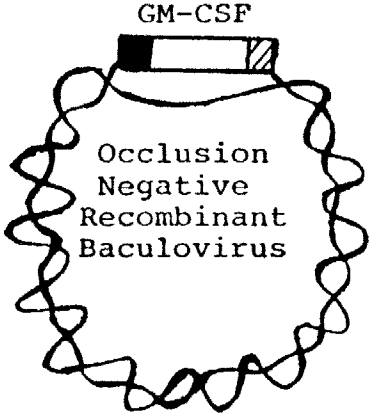
Calcium phosphate mediated transfection

↓

Sf9 cells

↓

Substitution of polyhedrin gene with hGM-CSF gene from pAc373GM-CSF by homologous recombination



↓

Transfection

↓

Sf9 cells

↓

Plaque hybridization

↓

Recombinant virus

↓

Assay biological activity using human cord blood cells

Purification of Viruses and Viral DNA from Infected Cells: Log phase Sf9 cells were centrifuged at 200-300g (500-600 rpm in a Beckman Model TJ-6 centrifuge) for 10 min. The supernatant was removed and the cells were resuspended in TNM-FH medium without FCS. One hundred mm plates were seeded with 6 x 10^6 cells in 9 ml of TNM-FH medium without FCS. The cells were allowed to attach to the plates at 27°C for 1 hr. After the cells were attached, the media was removed and 3 ml of virus inoculum (6 x 10^7 pfu/3 ml) was added to each plate. The plates were rocked to achieve an even virus distribution and incubated for 1 hr at 27°C. After this period, the inoculum was removed and 9 ml of TNM-FH medium was added to the plates. After 48 hr postinfection the supernatant containing ECV was collected and centrifuged at 400-500g (1500 rpm in a Beckman Model TJ-6 centrifuge) for 15 min to remove cell debris and polyhedra. The supernatant was transferred to ultracentrifuge tubes and centrifuged in a SW-27 rotor at 100,000g (24,000 rpm) for 30 min. The virus pellet was resuspended in 0.1X TE buffer and layered on 25%-56% sucrose gradients. The gradients were centrifuged at 100,000g (30,000 rpm) for 90 min at 4°C (SW-41 rotor). The virus band was removed, diluted with buffer, pelleted by centrifugation at 100,000g for 30 min at 4°C (again in a SW-41 rotor), and resuspended in 0.1X TE buffer. Proteinase K

was added to purified virus solution to a final concentration of 4 mg/ml and incubated for 2 hr at 50°C. Sarkosyl was added to a final concentration of 1% and incubated at 50°C for an additional 2 hr. Viral DNA was extracted with phenol and precipitated with ethanol as described earlier.

Transfection of Sf9 Cells: Plasmids containing the hGM-CSF gene were transferred to the AcNPV genome by recombination *in vivo*, using a modification of the calcium phosphate precipitation technique (Graham et al., 1973) as modified for insect cells (Burand et al., 1980; Carstens et al., 1980). After the Sf9 cells were seeded into a 25 cm^2 flask at a density of 2.0 x 10^6 cells for at least 1 hr, the media was removed and replaced with 0.75 ml of Grace's medium containing 10% FCS and antibiotics. Then, 0.75 ml of transfection buffer (25 mM HEPES, pH 7.1, 140 mM NaCl, 125 mM $CaCl_2$) was mixed with 1 µg of AcNPV DNA and 2 µg of plasmid DNA and added dropwise to the Grace's medium already in the cell culture flasks. The medium was removed after 4 hr incubation at 27°C. Then, the flasks were rinsed with fresh TNM-FH medium containing 10% FCS and antibiotics; 5 ml of TNM-FH medium were added, followed by incubation at 27°C for 4-5 days. At 4 or 5 days pi, collect the medium, remove the cells by centrifugation and store the virus-containing

supernatant at 4°C for further plaque hybridization.

Plaque Hybridization: 2 x 10^6 Sf9 cells were seeded into culture plates (60 x 15 mm) and incubated at 27°C for at least 1 hr. After the media was removed, 1 ml of serially diluted viruses was added into each plate. The viruses were distributed evenly by gently shaking plates before the plates were incubated at room temperature (or 27°C) for 1 hr. All inoculum was then removed and 4 ml of 1.5% low melting point agarose was slowly added to each plate. The plates were allowed to stand at room temperature for 1 hr to allow solidification before incubation at 27°C for 4-6 days (4 days). When the plaque form came out in the plates, the plates were left in an unhumidified environment to allow to dry overnight. The agarose and plate orientation were marked using a fine point marker. The agarose was transferred to a larger (100 x 20 mm) petri dish and filters were laid on top of the cells remaining in the plate. The filters were processed and hybridized with the cDNA of hGM-CSF. The 0.8 kb hGM-CSF fragment was isolated from an agarose gel as described earlier and labeled by a nick translation reaction (Maniatis et al., 1982). Labeling was done by incubating with 1 µg of the fragment, 200 pg DNase I, and 2 units DNA polymerase I in 50 µl of 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 20 µM each of dATP,dGTP, and dTTP, and about 1.5 µM of [α-

^{32}P] dCTP for 1 hr at 15°C. Stop buffer (30 mM Na_2EDTA pH 8.0) was added to the reaction after 1 hr incubation. Before use in hybridization, hGM-CSF cDNA probe was heated in a boiling water bath for 5 min and then quick-chilled in an ice bucket. After developing the autoradiogram of hybridized filters, agarose surrounding each positive area was transferred to a 48 well microtiter plate with 200 µl of Sf9 cells at a density of 1 x 10^5 cells/ml. After 3 days postinfection, each separate well was examined in order to determine whether the wild type viruses were contaminated or not using an inverted phase microscope and identified recombinant viruses by dot blot hybridization (Kafatos et al., 1979). If a significant number of cells had viral occlusions, plaque-hybridization was carried out again. The recombinant baculoviruses were identified and purified through several rounds of plaque hybridization.

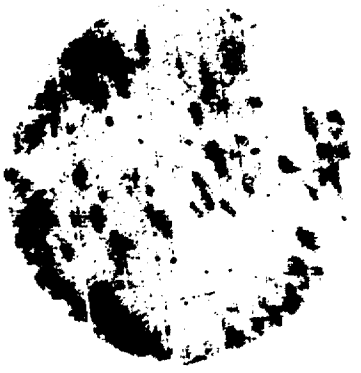
Results

The resulting chimeric transfer vectors pAc373GM-CSF were cotransfected into Sf9 cells with AcNPV viral DNA by the calcium phosphate precipitation technique. After 4-6 days postinfection, the cells were checked under an inverted microscope. The signs of infected cells include (1) appearance of polyhedra, (2) A 25-50% increase in diameter of

cells, (3) cell lysis late in infection (after 4-5 days). 10-50% of the cells have occlusions visible in the nucleus during 3-4 days pi and recombinant virus can account for 0.1-5% of the viral plaque. Recombinant virus was initially identified by DNA hybridization analysis of infected cells shown in Fig.11; later, screenings were performed visually, by the methods of occlusion negative plaque selection, i.e. those lacking the polyhedrin protein. The morphology of recombinant AcNPV-infected cells is quite different from uninfected and AcNPV-infected cells shown in Fig.12. Cells infected with wild type AcNPV virus show crystal-like viral occlusion in their nuclei using an inverted microscope. In comparison to AcNPV-infected and uninfected Sf9 cells, no crystal-like viral occlusions appear in the recombinant AcNPV-infected cells. It is also observed that vAc373GM-CSF-infected cells increase 25-50% in diameter relative to uninfected cells. The recombinant viruses were isolated by several rounds of plaque hybridization and visual screening. As shown in Fig.13, the diameter of a majority of infected cells became larger during 3-4 days the recombinant viruses infection.

Fig. 11. Screening of recombinant viruses Ac373GM-CSF. Autoradiogram of a Nylon filter prepared from a 60 mm plate containing plaques. The plaques were lysed and hybridized with a hGM-CSF cDNA. (A) primary screening of plaques (B) second screening of plaques from the area of the positive plaques shown in (A).

(A)



(B)

Fig. 12. Appearance of uninfected, AcNPV- and recombinant AcNPV-infected Sf9 cells. Supernatant medium from Sf9 cells cotransfected with wild type viral DNA and pAc373GM-CSF after 5 days was collected and used to infected Sf9 cells. (A) Uninfected (B) AcNPV infected or (C) vAc373GM-CSF infected cells are shown 72 hr postinfection.

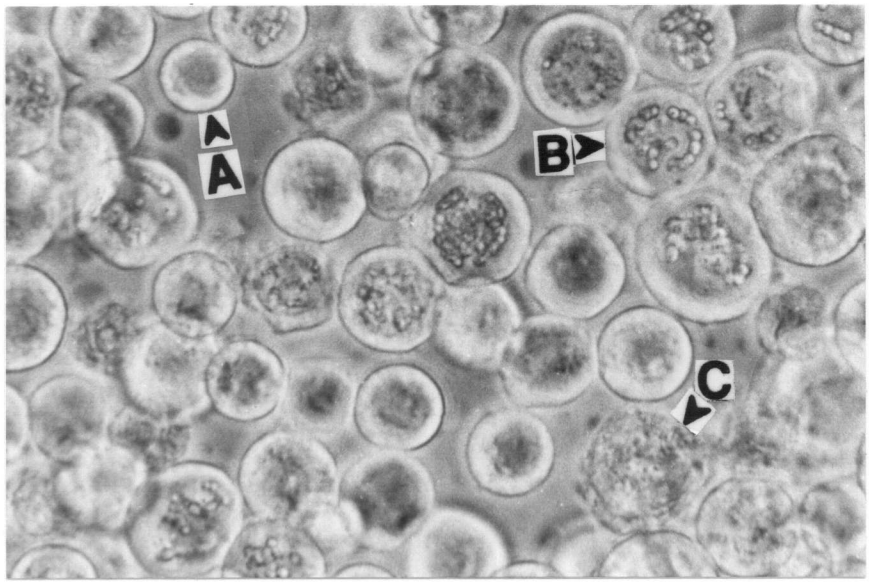
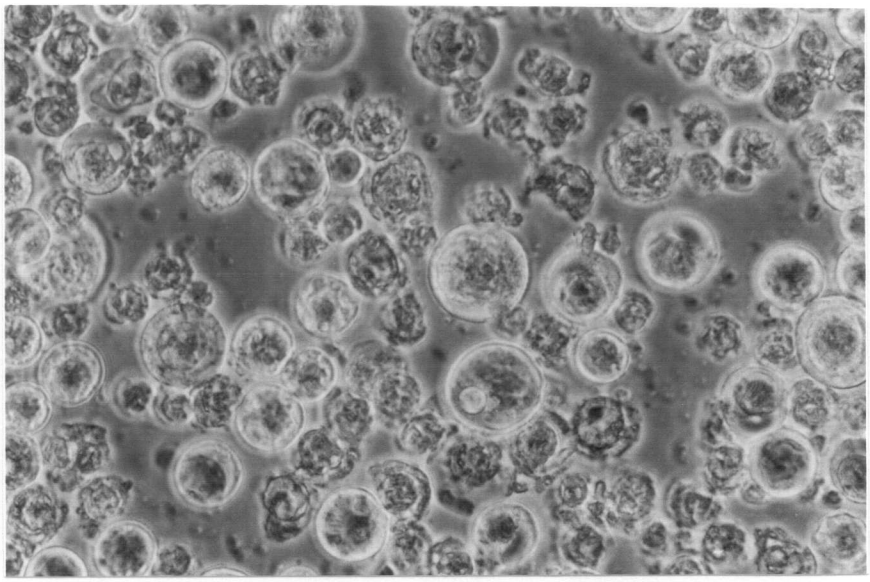

A
B
C

Fig. 13. Appearance of infected cells with recombinant viruses vAc373GM-CSF. vAc373GM-CSF infected Sf9 cells were shown at 72 hpi.



Discussion

It is advisable to produce high extracellular virus titers for viral DNA preparation from infected cells at an M.O.I. of 10 or greater. An M.O.I. of less than 1 will give less defective interfering particles when viral inoculum is prepared from the infected cells. Because viral DNA quality is important to the successful construction of recombinants, infection of Sf9 cells with viral DNA alone and analysis of viral DNA with restriction endonuclease is used to test the quality of a viral DNA preparation. After *Eco*RI digestion of viral DNA, more than 15 restriction fragments were present in 0.7% agarose gel and no smear bands were detected in agarose gel (data not shown). Deletion or insertional inactivation causes the production of occlusion negative viruses (Occ^-), which form plaques that are distinctly different from those of wild-type, occlusion positive viruses (Occ^+). The distinctive plaque morphologies can be the most rapid way to visually screen for recombinant viruses but may be difficult at first without previous experience. Plaque hybridization, therefore, could be an alternative approach. Although plaque hybridization requires more time to screen recombinant viruses, one of the major advantages of plaque hybridization over visual screening is identifying right recombinant

viruses instead of screening false Occ^- plaques. Three rounds of plaque hybridization are usually sufficient to isolate recombinant viruses. For production of biologically active recombinant hGM-CSF, recombinant virus in 48 wells were examined for (1) positive dot blot hybridization, (2) occlusion negative viruses and (3) biological activity of recombinant hGM-CSF.

CHAPTER V

IDENTIFICATION AND CHARACTERIZATION OF RECOMBINANT hGM-CSF

The recombinant viruses identified by plaque hybridization were examined to determine whether the recombinant hGM-CSF protein was being expressed. The conditioned medium of infected cells with these recombinant viruses was measured for hGM-CSF activity using human cord blood cell assay. A large stock of recombinant virus was prepared from culture media. The recombinant hGM-CSF in culture medium from infected cells was first concentrated with Amicon's ultrafiltration cell and then purified with Ultrogel AcA 44 gel filtration and Concanavalin A-Sepharose 4B affinity chromatography. The hGM-CSF fractions detected by dot blot immunoassay were analyzed using Western blot. The hGM-CSF proteins separated by electrophoresis and electroblotted onto membrane were sequenced directly in a gas-phase sequencer. Furthermore, the nature of the glycosylation in recombinant hGM-CSF was determined.

Materials and Methods

Bio-Dot microfiltration apparatus, mini Trans-Blot cell, nitrocellulose membrane, goat anti-rabbit horseradish peroxidase conjugate second antibody and color development agents were purchased from Bio-Rad, Richmond, CA. Amicon's disc membrane, and stirred cell were from Amicon Co., Danvers, MA. Polyclonal rabbit anti-hGM-CSF and N-Glycanase enzyme were from Genzyme, Boston, MA. Tunicamycin was purchased from Calbiochem, San Diego, CA.

Identification of hGM-CSF Produced by Recombinant Viruses: For measuring hGM-CSF activity expressed from the recombinant viruses, 2 ml conditioned medium (CM) of infected cells from a 60 mm plate was collected. The hGM-CSF activity of conditioned medium was measured using human cord blood cells assay as described earlier. Dot blot immunoassay is another assay to detect hGM-CSF produced by recombinant viruses. The CM was centrifuged at 100,000 g for 30 min at $4^{o}C$ to remove cells, debris and viruses. 100 µl of CM was directly loaded on a Bio-Dot microfiltration apparatus containing a nitrocellulose membrane prewet with TBS (Tris-Buffered Saline, 20 mM Tris-HCl pH 7.5, 500 mM NaCl). The samples were filtered through the membrane by gravity flow

for 1 hr. The wells were dried completely under vacuum. 100 µl of blocking solution (0.1% BSA in TBS) was added and gravity filtered for another 1 hr and vacuum dried completely. One hundred µl of rabbit anti-hGM-CSF antibody was added to wells and incubated at 4°C overnight. The incubation solution was filtered by gravity for 1 hr. Each well was washed with 200 µl TBS-Tween (0.05% Tween-20 in TBS) twice and drained completely. The filter was removed from the apparatus and transferred to TBS-Tween solution and washed for 10 min with shaking. After 1 hr incubation in TBS-BSA containing horseradish peroxidase (GAR-HRP) conjugate goat anti-rabbit IgG antibody, the filter was washed three times with TBS-Tween for 5 min each and once with TBS for 5 min. Color of filter was developed in 16% methanol, 83% TBS, 0.015% H_2O_2 and 0.5 mg/ml 4-chloro-1-naphthol.

The time course of hGM-CSF production in vAc373GM-CSF-infected cells was examined in order to detect the kinetic of hGM-CSF expression in the medium. For this, cells (2×10^7) were seeded in 100 mm tissue flasks and inoculated with either wild-type or recombinant viruses. The infected cells were washed twice with TNM-FH medium and then incubated at 27°C in 10 ml of TNM-FH medium. The media at 24, 48, and 72 hpi were assayed for biological activity using human cord blood cells. The presence of hGM-CSF-specific polypeptide

was also analyzed by Western blot analysis. The media at 24, 48 and 72 hpi were analyzed by SDS-PAGE with the discontinuous system of Laemmli (Laemmli, 1970). Gels were stained with either Coomassie blue or silver staining. For Coomassie blue staining, gels were stained with 0.2% Coomassie blue R-250 in 46% methanol and 8% acetic acid for 1 hr and destained with 5% methanol and 7% acetic acid overnight. The silver staining was performed as described (Oakley et al., 1980; Merril et al., 1981). Following electrophoresis the proteins were fixed by soaking the gel for 30 min with 40% methanol/10% acetic acid and for 15 min with 10% ethanol/5% acetic acid twice. The gel was then soaked in the 1X oxidizer of Bio-rad silver stain kit for 5 min. The gel was washed several times with deionized H_2O overnight prior to soak with 1X silver reagent for 20 min. The gel was rinsed with deionized H_2O for 1 min and soaked in developer until the desired level of staining was attained. Development was stopped by discarding the developer and adding 5% acetic acid. For Western analysis (Towbin et al., 1979), proteins separated by SDS-PAGE gel were electrophoretically transferred to nitrocellulose membranes with a mini Trans-Blot cell (Bio-Rad). After transfer, the membranes were rinsed with blocking buffer (TBS-Tween) and treated with 3% gelatin blocking buffer for 0.5 hr with

shaking at room temperature and probed overnight at 4°C with rabbit anti-hGM-CSF antibody at a dilution of 1:200 in 0.1% gelatin blocking buffer. The filters were washed three times with 0.1% gelatin blocking buffer for 5 min and treated for another 1 hr at room temperature with GAR-HRP conjugate goat anti-rabbit IgG antibody. The filters were washed and developed as described previously. For analysis of hGM-CSF activity from gel slices, the medium at 72 hpi was electrophoresed on a 12% SDS-PAGE gel. The gel was cut into 10 slices over the range from low molecular weight species (<14 KDa) to 27 KDa, resulting in gel slices 0.25 cm in length and 0.8 cm in width. Each slice was extracted overnight with PBS containing 1 mg/ml albumin. The samples were then dialyzed, sterilized and assayed for hGM-CSF activity.

Purification of the Expressed hGM-CSF Products: To produce hGM-CSF, 2 x 10^7 cells per 100 mm plate were infected with vAc373GM-CSF for 1 hr. The infected cells were washed once with TMN-FH medium (or PBS) and 5 ml TNM-FH was added. The culture was incubated for an additional 72 hr and the conditioned medium (CM) was harvested. The cells, debris and viruses were removed by centrifugation at 50,000 rpm (100,000 g) for 30 min at 4°C. An Amicon's ultrafiltration cell was used to concentrate the CM to about 1 ml. The AcA 44 gel

filtration column (3 x 120 cm) was pre-equilibrated with 0.01 M Tris-HCl pH 7.5 buffer, 0.3 M NaCl, 0.01% PEG, and 0.01% NaN_3. The fractions were collected as 200 drops/tube with a flow rate of 12 ml/hr. An UV monitor measured the protein concentration. Aliquots of 0.4 ml from every other fraction were then examined using dot blot immunoassay as described earlier.

The positive fractions from AcA 44 gel filtration chromatography were pooled, concentrated, and dialyzed overnight against concanavalin A (Con A) buffer containing 0.1 M sodium acetate, pH 6.0 with 0.5 M NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.02% NaN_3 and 0.02% PEG. The dialyzed sample was then applied to a Con A-Sepharose 4B column (1 x 6 cm) equilibrated with Con A buffer. The Con A column was washed with 40 ml of Con A buffer followed by 50 ml of elution buffer containing 0.5 M α-methyl-D-mannoside in Con A buffer. The flow rate was maintained at 20 ml/hr and 5 ml (120 drops/tube) fractions were collected. The breakthrough fractions and elution fractions were separately pooled and concentrated in Amicon, and then analyzed by Western blot. For ion exchange chromatography, samples from positive fractions of AcA 44 were applied to a DEAE-Sepharose column (1 x 10 cm). The column was pre-equilibrated with initial buffer (0.01 M Tris-HCl pH 7.8 containing 0.01% NaN_3 and

0.01% PEG). After loading 3 ml sample, 3 ml of the initial buffer was added to the column. Then a linear gradient of 0-0.2 M NaCl in the same buffer was applied and 5 ml fractions were collected. Aliquots of 0.1 ml from every other fraction were then examined using the dot blot immunoassay as described earlier.

Amino Acid Sequence of Recombinant hGM-CSF: The purified hGM-CSF used for amino acid sequence analysis was run on a 12% SDS-PAGE minigel (7 x 10 cm, 0.5 mm thick) and electroblotted to a polyvinylidene difluoride (PVDF) membrane according to published methods (Matsudaira, 1987). Briefly, after electrophoresis, the gel was soaked in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), 10% methanol, pH 11.0) for 10 min prior to transblotting (200 mA for 1 hr). The blotted PVDF membrane was washed with deionized H_2O for 5 min, stained with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min, and then destained for 10 min with 50% methanol-10% acetic acid. The membrane was finally rinsed in deionized H_2O for 5-10 min and air dried. hGM-CSF bands on PVDF membrane visualized by Coomassie Blue R-250 were excised with a razor blade and placed in the sequencer cartridge. Proteins were sequenced on an Applied Biosystems model 475A sequenator (Hewick et al., 1981). The

identification of phenylthiohydantoin (PTH)-amino acid derivatives was carried out by reverse-phase HPLC over a Brownlee C-18 column.

Glycosylation Analysis of Expressed hGM-CSF Product: Sf9 cells infected with vAc373GM-CSF at 14 hpi were treated with 5 μg of tunicamycin per ml in order to determine the effect of glycosylation in Sf9 cells. After 3 days infection, the conditioned medium was collected and analyzed by Western blot. Further, to determine directly if hGM-CSF undergo N-glycosylation in insect cells, the glycosylated proteins (600-700 μg/ml) at Con A stage purification were adjusted to 0.5% SDS and 0.1 M β-mercaptoethanol and boiled for 2 min. The disrupted sample was diluted threefold with 200 mM sodium phosphate (pH 8.5). N-glycanase was added to a final concentration of 1-10 U/ml. After incubation for at least 16 hr at 37°C, the sample was analyzed by SDS-PAGE and Western blot.

Results

Media samples from recombinant virus vAc373GM-CSF-infected cells have detectable hGM-CSF activity shown in Table III. The isolation of the recombinant viruses was further demonstrated by their ability to produce and secrete

TABLE III

HGM-CSF ACTIVITY BY RECOMBINANT VIRUSES INFECTED CELL SUPERNATANTS

Source	Colonies per 2 x 10^5 human cord blood cells		
	Amount of supernatants		
	10 µl	50 µl	200 µl
original			
pAcA373GM-CSF + wild type (WT) Virus	57	65	20
pVL941GM-CSF + WT Virus	69	66	78
pAc360β-galactosidase + WT Virus	2	5	5
second screening			
16-1 (vAc373GM-CSF)	26	46	52
16-3 (vAc373GM-CSF)	35	31	59
19-7 (vVL941GM-CSF)	18	14	20
19-8 (vVL941GM-CSF)	58	57	53
WT Virus	2	5	4
H_2O	—	—	0
MIA PaCa-2	—	52	89

hGM-CSF into the medium. Condition medium from insect cells infected with recombinant viruses was assayed for hGM-CSF activity in Fig.14. hGM-CSF activity in the medium was low, but detectable at 24 hpi, and steady accumulation of hGM-CSF is observed up to 72 hpi. Assuming a hGM-CSF specific activity of 1-4 x 10^7 U/mg (Wong et al., 1985), the highest accumulation of hGM-CSF in the medium (72 hpi) corresponds to an expression of 11-45 μg/ml (10^6 cells/ml). Media samples from uninfected cells and cells infected with AcNPV had no detectable hGM-CSF activity. Conditioned medium of infected cells at 24, 48 and 72 hpi was concentrated and run on SDS-PAGE, and proteins detected by Coomassie blue staining and Western blot analysis using anti-hGM-CSF antibody (Fig.15). Three proteins reacting with hGM-CSF antibody were detected to have accumulated in the medium by 48 hpi, of molecular weights 14.5, 15.5 and 16.5 KDa. This appears to suggest the expression of three different forms of hGM-CSF in the infected cells. The SDS-PAGE gel was cut and the gel slices assayed for hGM-CSF activity (Fig. 16). Biological activity was found to be associated with gel slices over the range from 14 to 17 KDa, suggesting that three species of hGM-CSF are biologically active.

Serum-free conditioned medium of hGM-CSF was prepared in 100 mm plates containing vAc373GM-CSF-infected cells. Conditioned medium was concentrated by Amicon and hGM-CSF was

Fig. 14. Time course of hGM-CSF activity in Ac373GM-CSF infected Sf9 cells. Cells were infected with vAc373GM-CSF at a cell density of 10^6 cells per ml. At 24, 48 and 72 hr postinfection, samples were taken and the cells were removed by centrifugation. Media were assayed for hGM-CSF biological activity using human cord blood cells. The hGM-CSF activity in the media of vAc373GM-CSF-(■) and AcNPV-(◆) infected cells was described in the methods.

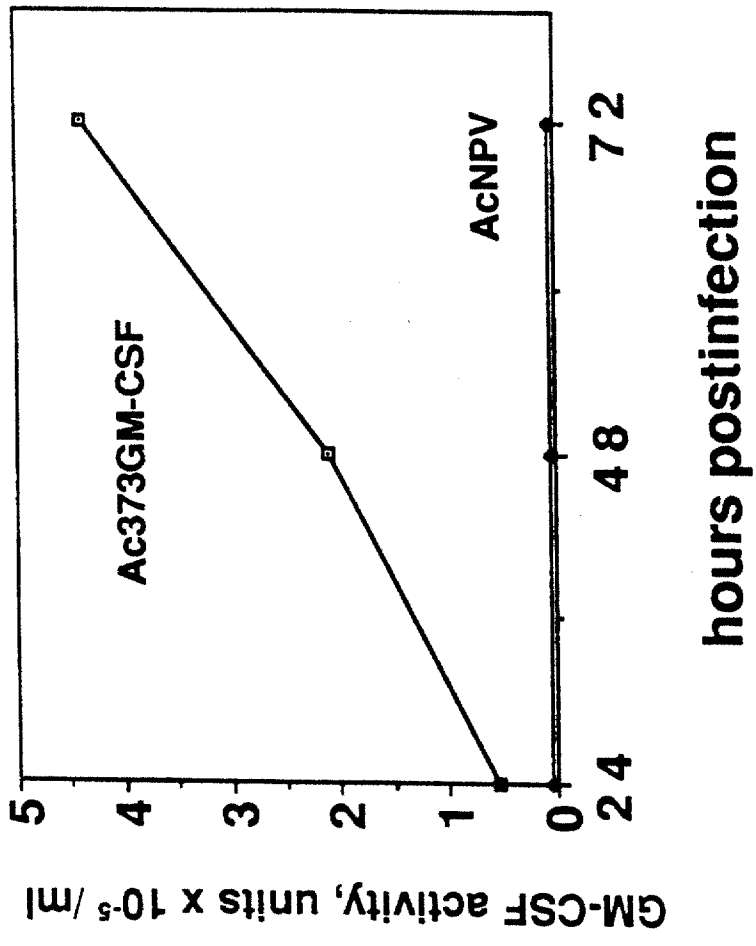

GM-CSF activity, units x 10-5 /ml
5
4
3
2
1
0
2 4
4 8
7 2
hours postinfection
Ac373GM-CSF
AcNPV

Fig. 15. SDS-PAGE of hGM-CSF release in the medium of vAc373GM-CSF infected cells. Cells were infected with vAc373GM-CSF at a cell density of 10^6 cells per ml. At 24, 48 and 72 hpi, media were electrophoresed on a 12% SDS-PAGE gel and electrophoretically transferred to nitrocellulose for immunoblot analysis. Lane 1, lane 2 and lane 3 represent 24, 48 and 72 hpi in (a) Coomassie blue stain and (b) Western blot. Lane M is protein standards.

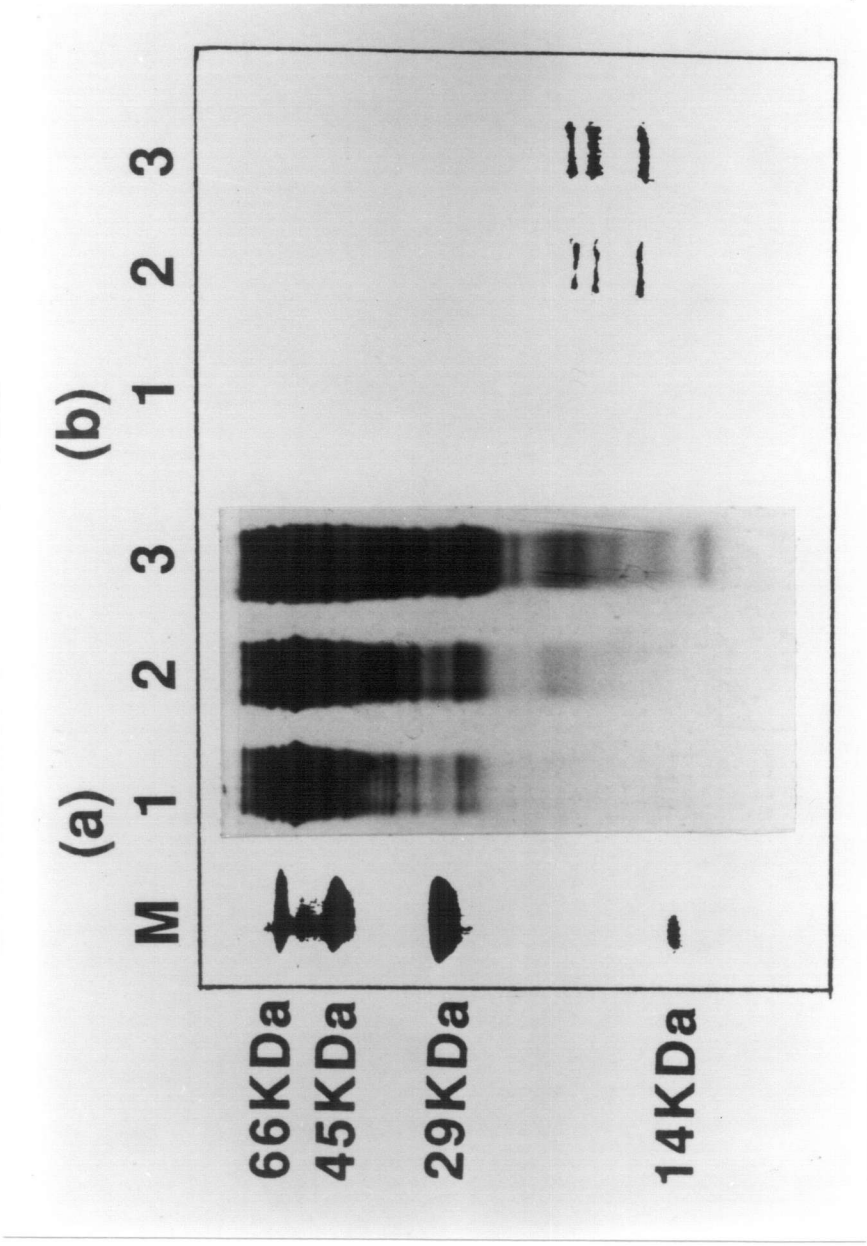
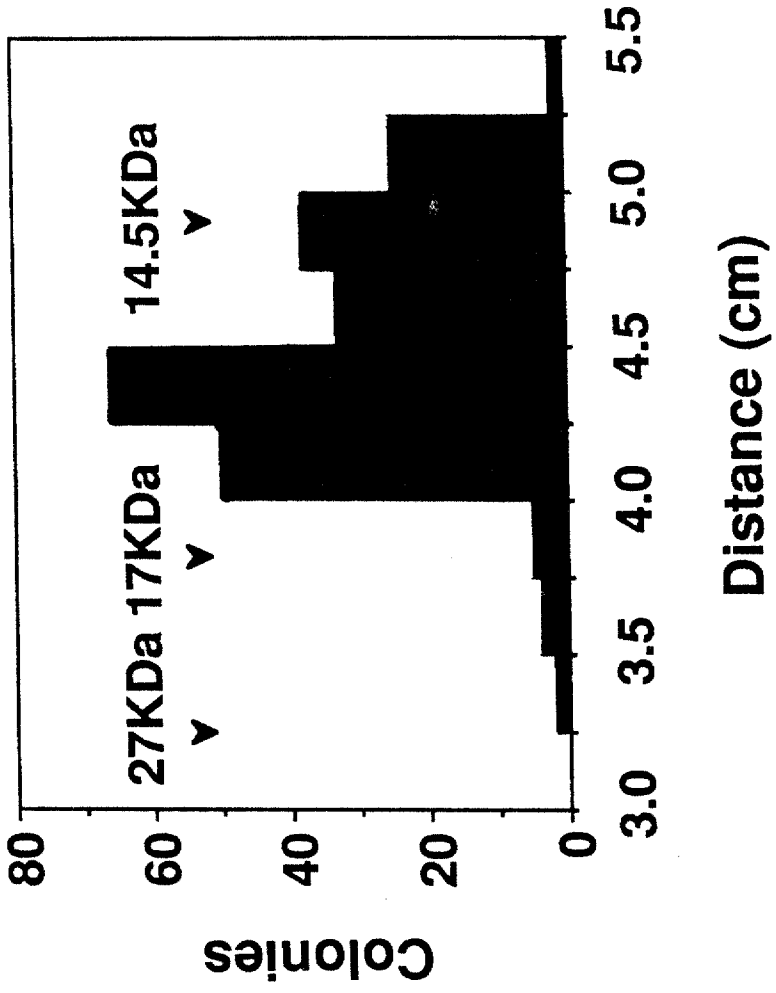

(a)
(b)
M
1
2
3
1
2
3
66KDa
45KDa
29KDa
14KDa

Fig. 16. hGM-CSF activity profile on SDS-PAGE. Conditioned medium from vAc373GM-CSF infected cells was analyzed on a 12% SDS-PAGE gel. The gel was sliced and assayed for hGM-CSF activity as described in the methods.


Colonies
80
60
40
20
0
27KDa
17KDa
14.5KDa
3.0
3.5
4.0
4.5
5.0
5.5
Distance (cm)

partially purified by AcA 44 gel filtration. In Fig.17, the fractions collected from chromatography of conditioned medium on AcA 44 were checked by dot blot immunoassay and the hGM-CSF peak came out in fractions 41 to 45. Since the hGM-CSF was eluted with a low amount of protein, this provided a good purification step. As shown in Fig.18, after AcA 44 gel filtration most of the proteins were separated from hGM-CSF. The hGM-CSF positive fractions from AcA 44 were pooled, concentrated and dialyzed against Con A column buffer for further Con A and DEAE purification. The fractionation of hGM-CSF on Con A-Sepharose chromatography is shown in Fig.19. The nonglycosylated proteins broke through the Con A column (fractions 1-4). The glycosylated proteins bound the Con A column and were eluted by α-methyl-D-mannoside (fraction 5-10). The Mr 14.5 KDa protein broke through Con A and the Mr 15.5 KDa and 16.5 KDa proteins bound to Con A were immunoreacted with hGM-CSF antibody in Fig.20. The fractionation of hGM-CSF on DEAE CL-6B is illustrated in Fig.21. The positive fractions of hGM-CSF were eluted at 0.12 to 0.13 M NaCl wash which was similar to previous report of nature hGM-CSF elution profile (Wong et al., 1985). Fractions from Con A column that contained glycosylated hGM-CSF were pooled, run on SDS-PAGE gel and transblotted to PVDF membrane as described in Methods. As seen from silver-

Fig. 17. Gel filtration on Ultrogel AcA 44. Concentrated sample (12 ml, 360 mg) from the infected insect cell supernatant of vAc373-GM-CSF was loaded on Ultrogel AcA 44 column (3 x 100 cm). Samples (400 µl) from every other fraction (10 ml/tube) were examined by immunoassay. Absorbance at 280 nm, ▫ ; positive signal of immunoassay, ●.

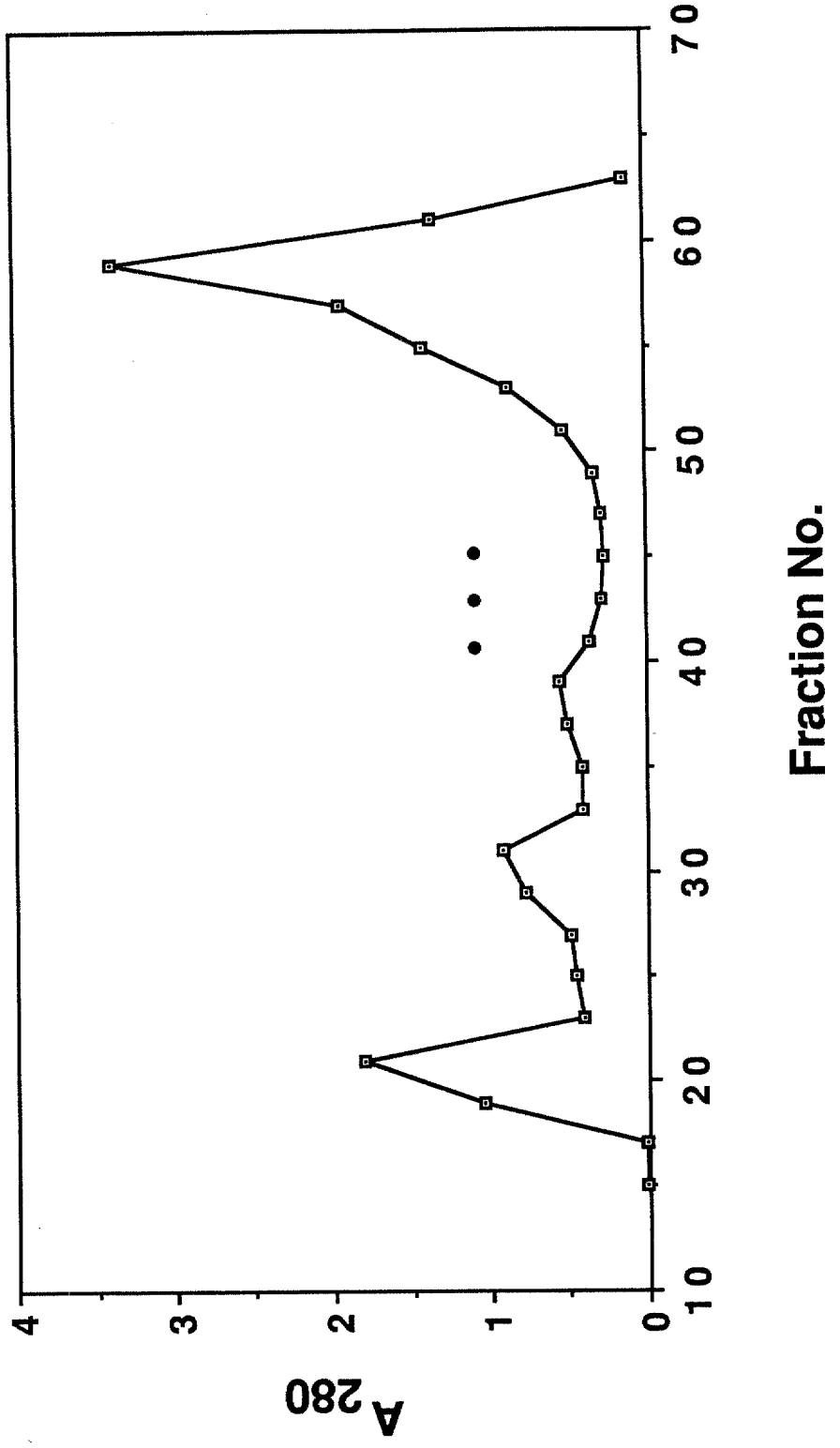

A 280
0
1
2
3
4
10
20
30
40
50
60
70
Fraction No.

Fig. 18 SDS-PAGE of proteins before and after AcA 44 gel filtration. Proteins from vAc373GM-CSF-infected cells at 72 hpi were electrophoresed on a 12% SDS-PAGE gel and electrophoretically transferred to nitrocellulose for immunoblot analysis. (a) Lanes 1 and 2: Coomassie blue-stained proteins before and after AcA 44. (b) Lanes 1 and 2: Western blot analysis prepared from (a) lanes 1 and 2, respectively. Lane M: molecular weight markers.

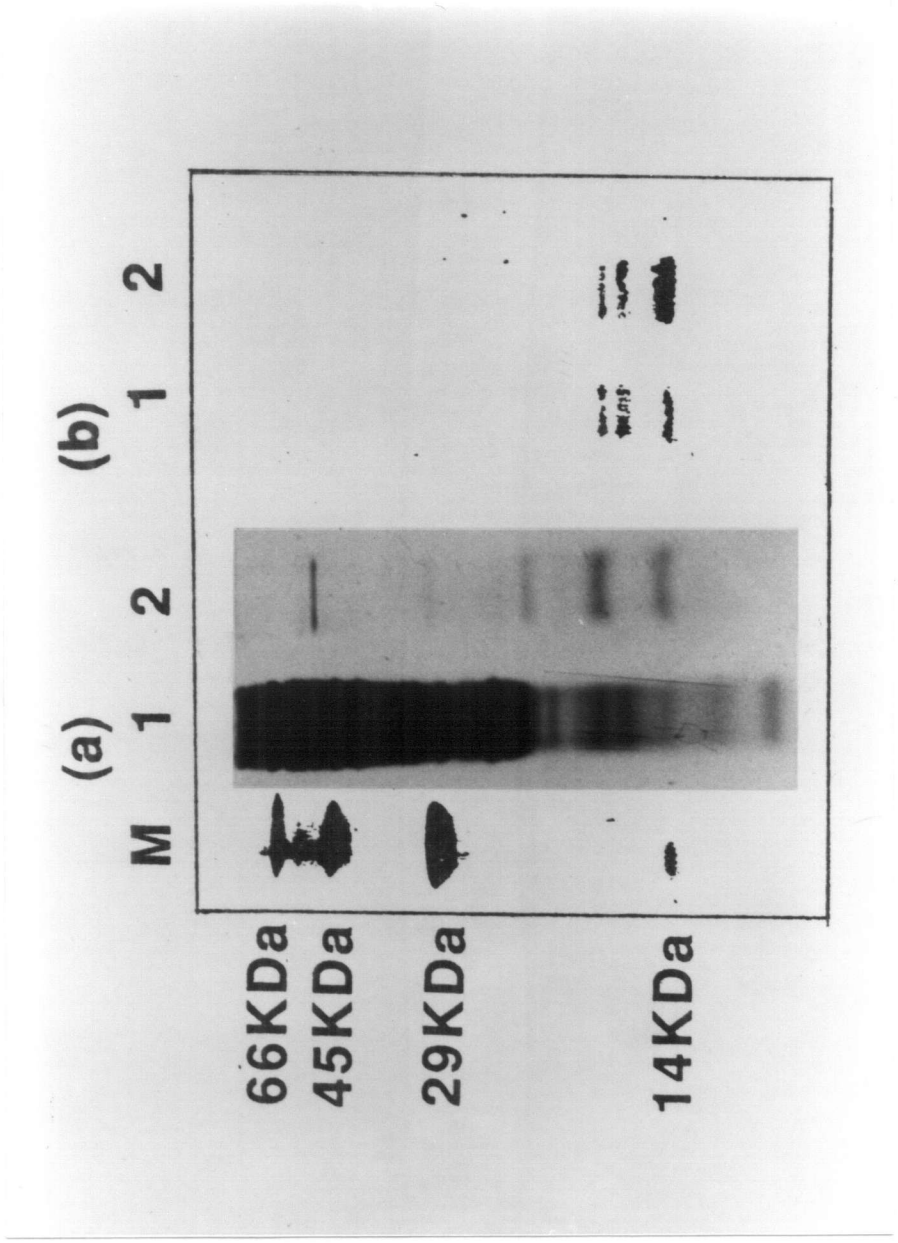

M
(a)
1
2
(b)
1
2
66KDa
45KDa
29KDa
14KDa

Fig. 19. Fractionation of hGM-CSF on concanavalin A Sepharose 4B. The positive fractions from the AcA44 chromatography were pooled, concentrated, replaced by the Con A column buffer and subjected to Con A chromatography as described in materials and methods. Absorbance at 280 nm, ▫.

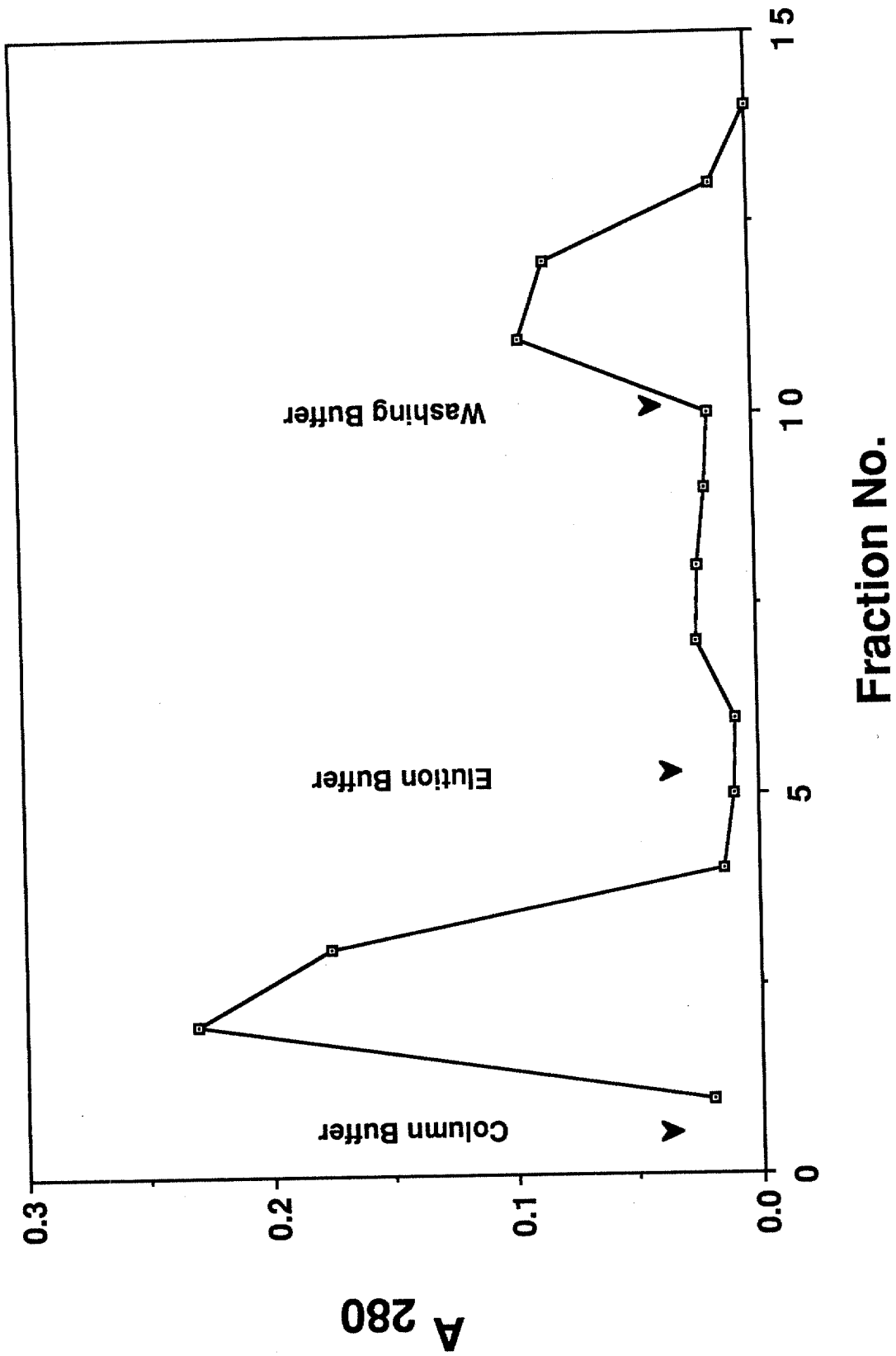

A 280
0.3
0.2
0.1
0.0
0
5
10
15
Fraction No.
Column Buffer
Elution Buffer
Washing Buffer

Fig. 20. SDS-PAGE of purified proteins from Con A affinity chromatography. Fractions from 1 to 5 and from 6 to 10 were pooled and concentrated, respectively. Samples from two concentrated fractions were electrophoresed on a 12% SDS-PAGE gel and electrophoretically transferred to nitrocellulose for immunoblot analysis. Lanes 1 and 2: Silver stained proteins from fractions 1 to 5 and from fractions 6 to 10 respectively. Lanes 3 and 4: Western blot analysis prepared from lanes 1 and 2, respectively. Lane 5: molecular weight markers.

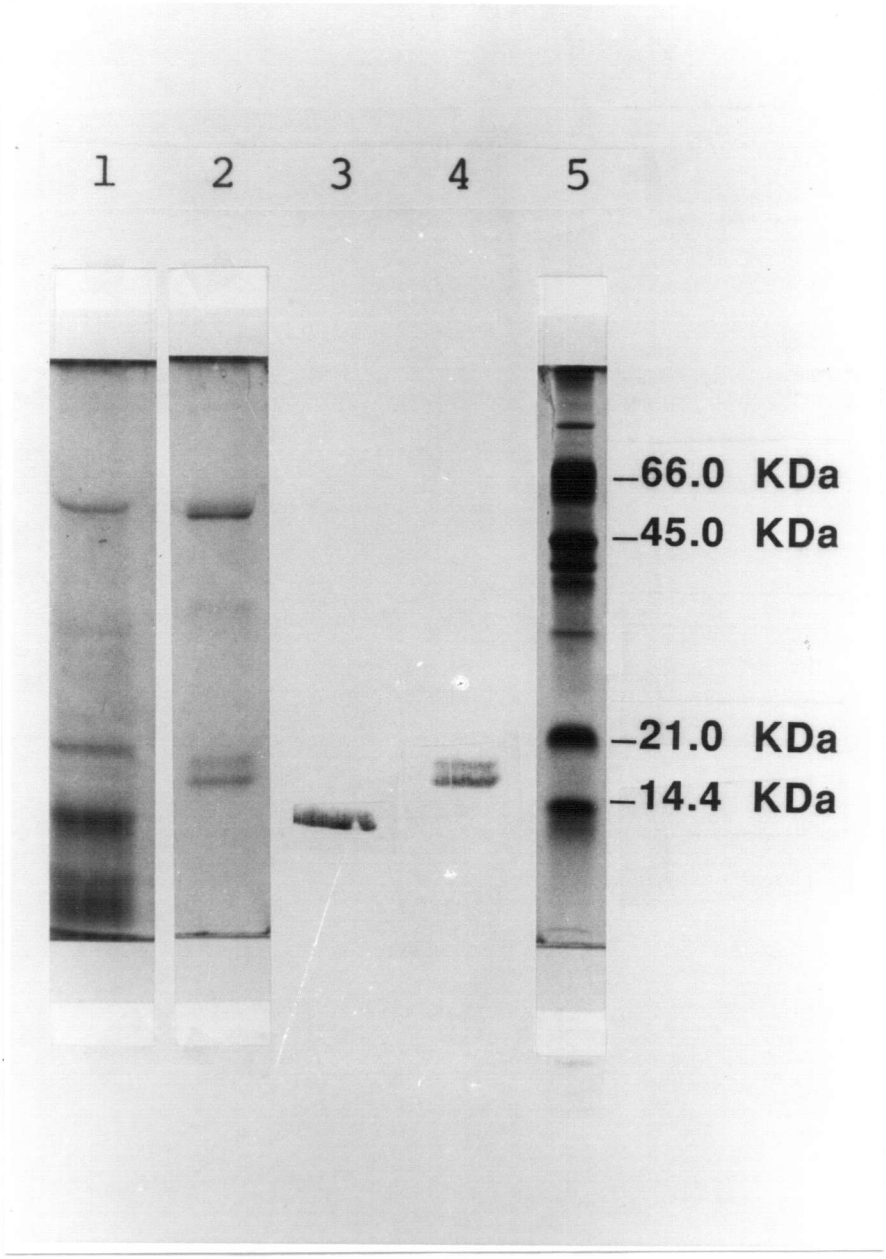
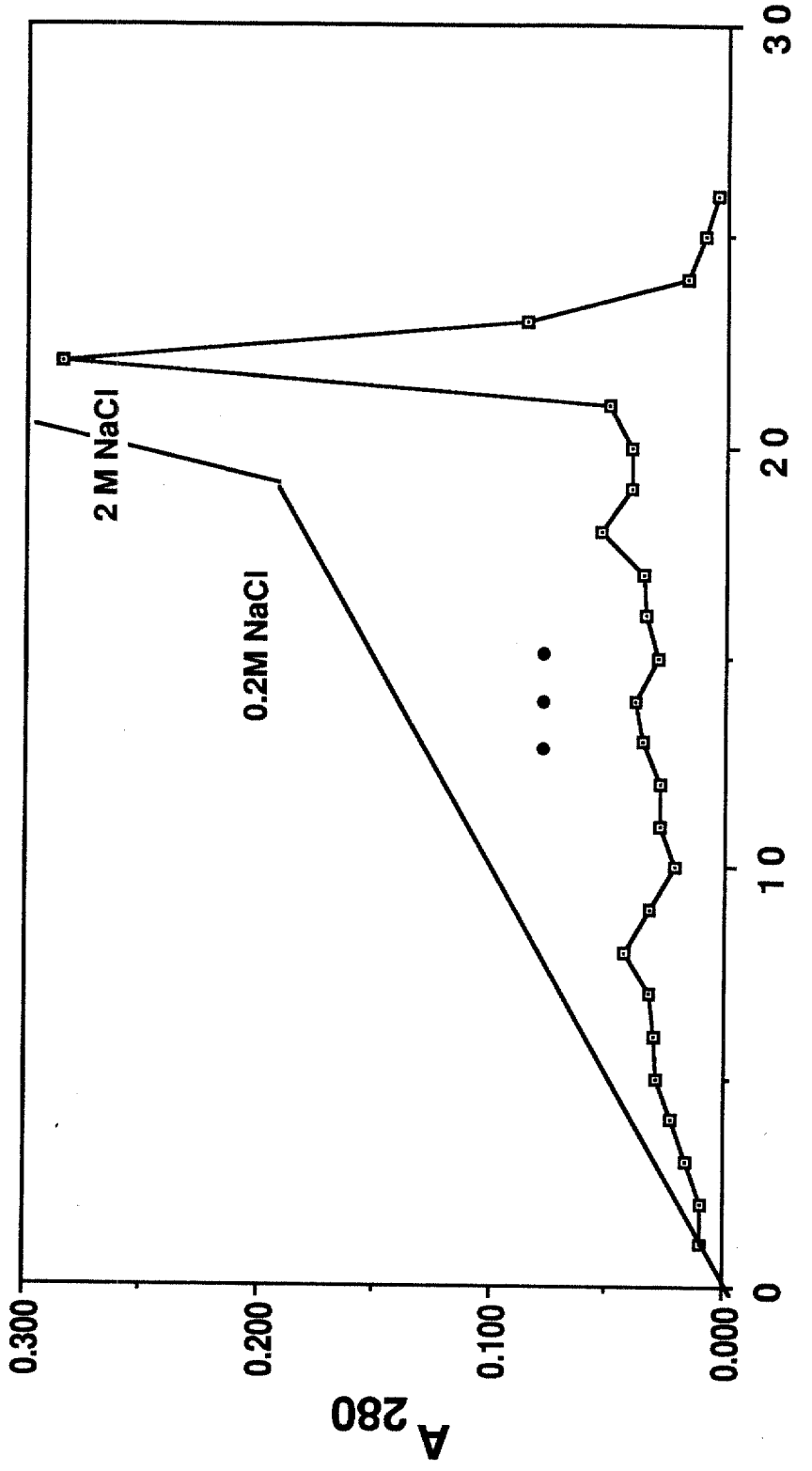

1
2
3
4
5
66.0 KDa
45.0 KDa
21.0 KDa
14.4 KDa

Fig. 21. Fractionation of hGM-CSF on DEAE CL-6B Sepharose. The positive fractions from the AcA 44 chromatography were pooled, concentrated, dialyzed and subjected to DEAE CL-6B chromatography. Every fraction was examined by immunoassay. Absorbance at 280 nm, ▫ ; positive signal of immunoassay, ● .


0.300
0.200
0.100
0.000
A 280
0
10
20
30
Fraction No.
2 M NaCl
0.2M NaCl

stained SDS-PAGE gel two hGM-CSF bands (Fig.20) confirmed by Western blot were sequenced. The results obtained from sequencing of recombinant hGM-CSF were shown in Fig.22. The amino-terminal sequence of two glycosylated hGM-CSFs from the Sf9 cell culture medium was identical to that of the nature hGM-CSF.

Tunicamycin, a inhibitor of all N-glycosylation in glycoprotein biosynthesis (Takatsuki et al., 1975), was used to determine the effect of glycosylation on the size of the hGM-CSF in Sf9 cells. In the presence of tunicamycin, the 15.5 and 16.5 KDa hGM-CSF glycoproteins were missing and only the 14.5 KDa hGM-CSF was shown by Western analysis as shown in Fig.23. However, tunicamycin had little effect on the production of the 14.5 KDa hGM-CSF protein in vAc373GM-CSF-infected cells. Furthermore, when the hGM-CSF glycoproteins were treated with N-glycanase, the 15.5 and 16.5 KDa of hGM-CSF was converted to a single species with Mr of 14.5-15.5 KDa as shown in Fig.24. The reduction of molecular weight of both bands by N-glycanase treatment indicated that both bands are N-glycosylated protein. However, the microheterogeneity of these two proteins remain to be further characterized.

Fig. 22. Amino-terminal sequence of recombinant hGM-CSF in the Sf9 cells. The mammalian peptidase cleavage site is indicated by the arrow and the first amino acid of mature protein is alanine. In the recombinant sequences, the blank (-) at positions 1 and 4 could not be decided alanin, glycine and serine.

(A) hGM-CSF deduced from cDNA

signal peptide												
	1	2	3	4	5	6	7	8	9	10	11	12
Met Trp___Ill Ser	Ala	Pro	Ala	Arg	Ser	Pro	Ser	Pro	Ser	Thr	Gln	Pro

(B) Recombinant hGM-CSF from Sf9 cells

upper band	-	Pro	Ala	-	Ser	Pro	Ser	Pro	Ser	Thr	Gln	Pro
lower band	-	Pro	Ala	-	Ser	Pro	Ser	Pro	Ser	Thr		
							Trp		Leu			

Fig. 23. Effect of tunicamycin (TM) on hGM-CSF secretion from Sf9 cells. vAC373GM-CSF-infected Sf9 cells were treated with 5 µg/ml tunicamycin at 14 hpi. At 72 hpi, samples were electrophoresed on a 12% SDS-PAGE gel and electrophoretically transferred to nitrocellulose for immunoblot analysis. Lanes 1 and 2: Western blot analysis prepared from proteins with and without TM treatment, respectively. Lane 3: molecular weight markers.

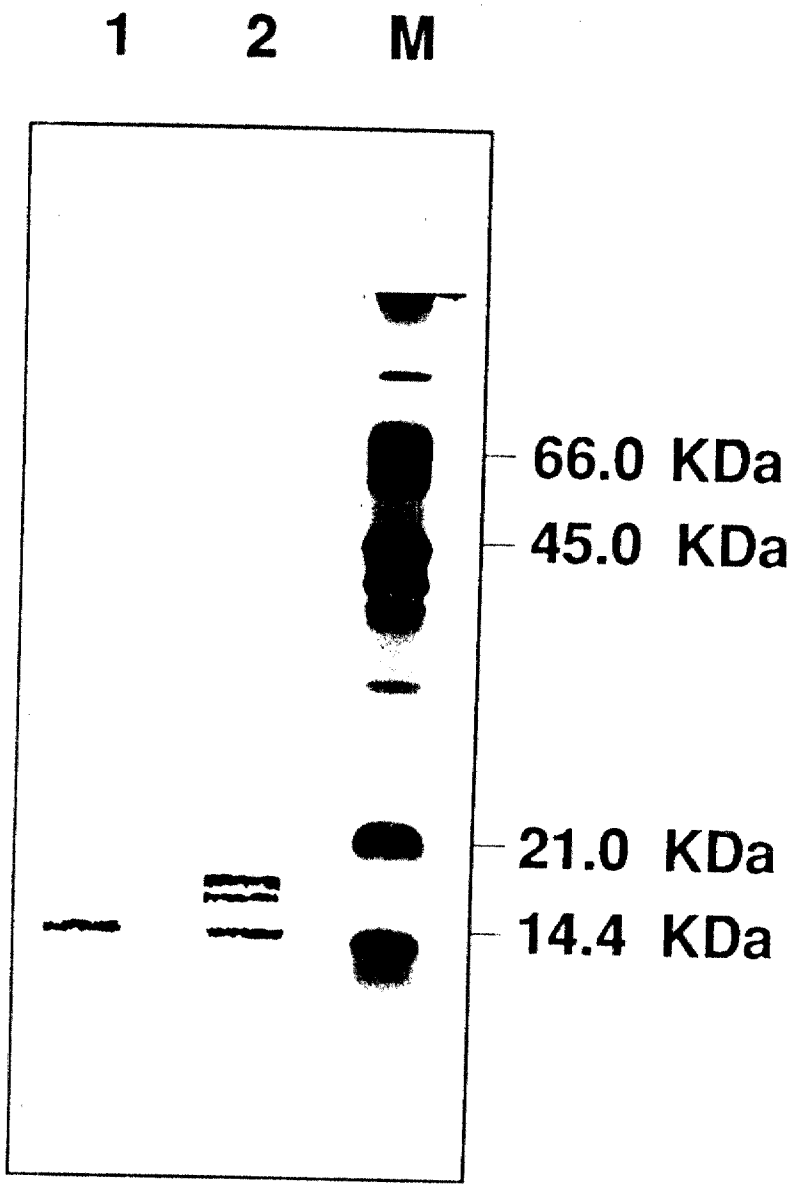
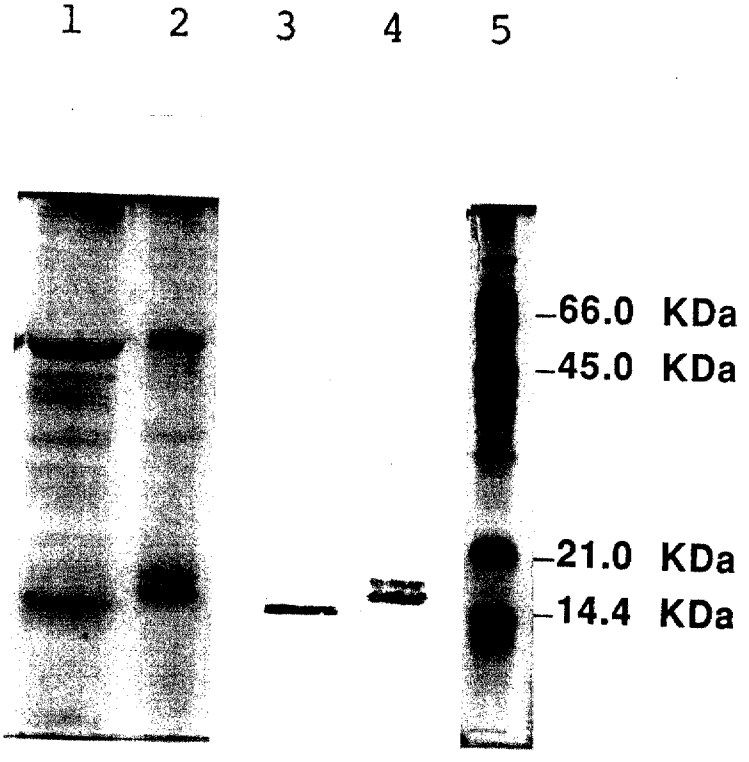

1
2
M
66.0 KDa
45.0 KDa
21.0 KDa
14.4 KDa

Fig. 24. N-glycanase analysis of insect cell-derived hGM-CSF. The glycosylated proteins at Con A stage purification were treated with N-glycanase as described in methods. The reaction products were analyzed by SDS-PAGE and Western blot. Lanes 1 and 2: Silver-stained proteins with and without N-glycanase treatment respectively. Lanes 3 and 4: Western blot analysis prepared from lanes 1 and 2, respectively. Lane 5: molecular weight markers.


1
2
3
4
5
66.0 KDa
45.0 KDa
21.0 KDa
14.4 KDa

Discussion

The hGM-CSF in the vAc373GM-CSF-infected cell supernatant could be identified either by biological assay or by immunoassay. According to the bioassay of hGM-CSF activity, high-level (11-45 μg/ml) expression of recombinant hGM-CSF into the infected cell medium occurs. Immunoassay, unlike the time consuming biological assay, could easily be used to detect the secreted hGM-CSF in the medium during protein purification. The purification of hGM-CSF from the conditioned medium of infected insect cells was carried out by AcA 44 gel filtration and Con A affinity chromatography. By this two-step procedure, glycosylated hGM-CSF could be separated from other proteins for further protein sequencing. In the silver stain and Western blot analysis of α-methyl-D-mannoside washing fractions from Con A column, only two glycosylated hGM-CSFs migrated at molecular weights of 15-17 KDa. After separated by electrophoresis and electroblotted onto PVDF membrane, the two hGM-CSFs were visualized by Coomassie Blue and sequenced directly in the gas-phase sequencer. The partial amino acid sequence of recombinant hGM-CSF showed that Mr 16.5 and 15.5 KDa were identical to natural hGM-CSF deduced from cDNA. These results demonstrated that these two forms of glycosylated hGM-CSF

were considered to be differentially posttranslationally modified forms of the same polypeptide.

CONCLUSION

Although different procaryotic and eucaryotic expression systems have been used to produce large quantities of proteins from cloned genes in the past few years, the structure and biological activity of the recombinant gene products are not always identical to the natural proteins. In the baculovirus-Sf9 cell expression system, a number of academic and commercial laboratories have successfully obtained abundant quantities of biologically active products from a variety of heterologous genes. The purpose of this research project is to use the Baculovirus Expression Vector System (BEVS) for human GM-CSF production in insect cells. Thus, a recombinant virus derived from the transfer vector pAc373 was constructed using a hGM-CSF cDNA. The cDNA insert was expressed, translated, and processed to recombinant hGM-CSF protein. The recombinant hGM-CSF from the insect cells described here and from other sources is summarized in Table IV.

Bioassay of cell supernatants, stimulating the formation of granulocyte and macrophage colonies from human progenitor cells, suggest that insect cells infected with a recombinant virus encoding the hGM-CSF recognize and cleave hGM-CSF

TABLE IV

COMPARISON OF RECOMBINANT HGM-CSF FROM THE INSECT CELLS AND OTHER SOURCES

Source	Molecular weight(KDa)	Glycosylation	Signal sequence cleavage	Secretion	Reference
COS-1	18-28	yes	yes	yes	Wong et al., 1985
CHO	16-27	yes	yes	yes	Moonen et al., 1987
Sf9	14-17	yes	yes	yes	in this study
Yeast	(a)	yes	(b)	yes	Miyajima et al., 1986
E.coli	14.5	no	(c)	(d)	Libby et al., 1987

a. The fused recombinant hGM-CSF proteins are detected in the yeast medium.

b. The yeast secretion signal of the α-factor mating pheromone is used in yeast expression vector.

c. The *E.coli* signal peptide of the outer membrane protein (ompA) is fused to the N-terminal of hGM-CSF DNA.

d. The recombinant hGM-CSF is secreted into periplasmic space of the *E.coli*.

signal sequence that directs the protein to the endoplasmic reticulum (ER) and secrete it into the culture medium.

In the Western blot analysis, hGM-CSFs secreted into the culture medium had apparent molecular weight of 14.5, 15.5 and 16.5 KDa. The two larger proteins were bound to the Con A column and eluted by α-methyl-D-mannoside, suggesting that hGM-CSF could be glycosylated in Sf9 cells. Further, treatment of glycosylated hGM-CSF with N-glycanase resulted in a decrease in the apparent molecular weight of the higher molecular weight species to approximately 14.5-15.5 KDa, confirming the presence of N-linked glycosylation. hGM-CSF has two potential N-linked glycosylation sites based on examination of the amino acid sequence derived from the cloned cDNA, and the results here suggest that two glycosylated forms are expressed in Sf9 cells. The two larger bands result from glycosylation heterogeneity as evidenced by the fact that a single band appears when these glycosylated proteins were treated with N-glycanase and infected Sf9 cells were treated with tunicamycin. All three different hGM-CSF forms secreted by insect cells show hGM-CSF activity, suggesting that difference exist in the posttranslational processing of the gene product resulting in three distinct but biologically active forms of the hGM-CSF. The difference in glycosylation of expressed hM-CSF

(Maiorella et al., 1988),HA (Kuroda et al., 1986) and mouse IL-3 (Miyajima et al., 1987) also occurs in the Sf9 cells. The previously reported size of the unglycosylated form of hGM-CSF in *E.coli* (Libby et al., 1987), and the fact that infected cells under tunicamycin treatment produced a single form at 14.5 KDa, suggests that the molecular weight of 14.5 KDa corresponds to the unglycosylated hGM-CSF.

The results of this research demonstrate that hGM-CSF protein is expressed at a level of 11-45 mg/L by infected Sf9 cells. Although it is lower than the viral protein of polyhedrin (1200 mg/L), it is comparable to the expression of some recombinant proteins in this system such as c-*myc* (Miyamoto et al., 1985), epidermal growth factor receptor (Greenfield et al., 1988) and interleukin 2 (Smith et al., 1985). At this level of expression an adequate quantity of functional hGM-CSF protein can be obtained for further physical-chemical analysis. In a comparative study of oligosaccharide processing, it seems that N-linked targeted sites for glycosylation in insect cell are the same as those of mammalian cells (Hsieh et al., 1984). However, the processing of the oligosaccharides at these sites differs in insect- and mammal-derived proteins. Mammalian cells extensively modify the core oligosaccharide in terminal glycosylation events involving the transfer of glucosamine-

galactose and sialic acid residues to form complex oligosaccharides. In insect cells, like mosquito and fruitfly, the cotranslational addition of a typical high-mannose oligosaccharide, $GlcNAc_2$-Man_9-Glc_3, is followed by the processing of about half of the total N-linked glycans to a trimannosyl core, (Asn)-$GlcNAc_2$-Man_3 (Shieh et al., 1984). Further processing of the oligosaccharide does not appear to take place, owing to the absence of the galactose and sialic acid transferase (Butters et al., 1981). The trimannosyl core probably represents the fully processed oligosaccharides on the extracellular form of hGM-CSF if Sf9 cells also lack these glycosyltransferase activities. Whether this is generally the case remains to be demonstrated.

Many other mammalian glycoproteins expressed in insect cells, for example the human β-interferon (Smith et al., 1983), human EGF receptor (Greenfield et al., 1988) and heamagglutinin of influenza virus (Kuroda et al., 1986), are synthesized as smaller glycoproteins than their naturally occurring counterparts. The complex type oligosaccharides commonly occurring in glycoproteins of vertebrate cells have not been found in insect cell (Hsieh et al., 1984). Instead, a much shorter and simpler oligosaccharide consisting of (Asn)-GlcNAc2-Man3 was commonly present in insect cell glycoprotein. In characterizing the oligosaccharide nature

of the product expressed in infected-Sf9 cells, we observed that N-glycanase treatment of the hGM-CSF hydrolyzed the oligosaccharides from the hGM-CSF, reducing the Mr 15.5 and 16.5 KDa forms to the size of 14.5-15.5 KDa, presumably the core protein. The small change of molecular weight suggests that the hGM-CSF expressed lacks complex type oligosaccharide modifications. The molecular weight of hGM-CSF expressed in this system of 14-17 KDa is comparable to that of 22 KDa for hGM-CSF produced by Mo cells (Wong et al., 1985) which would be expected to contain complex type oligosaccharide modifications. Additional work will be required to further characterize the hGM-CSF oligosaccharide modifications observed in this study.

In conclusion, hGM-CSF protein is expressed at high levels in insect cells under the strong polyhedrin promoter. In addition, the pre-GM-CSF signal peptide could be recognized and cleaved within the insect cells and the recombinant hGM-CSF is secreted into the media during infection. Importantly, hGM-CSF produced in insect cell is able to stimulate colony formation in cord blood cell assay. Thus, the recombinant virus-produced hGM-CSF obviously resembles authentical hGM-CSF.

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