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Expression of human granulocyte-macrophage colony-stimulating factor gene in insect cells by a baculovirus vector

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A plasmid pAc373GM-CSF was constructed and co-transfected into *Spodoptera frugiperda* (Sf9) cells with wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA. The recombinant virus vAc373GM-CSF was identified and purified by several rounds of plaque hybridization. By assaying the culture medium, we demonstrated recombinant virus infected Sf9 cells expressing hGM-CSF. Recombinant hGM-CSFs with apparent molecular masses of 14.5, 15.5 and 16.5 kDa were detected by the Western blot method. All 3 forms have biological activity of hGM-CSF. Following *N*-glycanase treatment, a single band of 14.5–15.5 kDa appeared in SDS-PAGE. Western blot analysis of expression in Sf9 cells treated with tunicamycin revealed only the presence of the 14.5 kDa species. Thus, the signal sequence of recombinant hGM-CSF could be recognized and cleaved by infected insect cell and the resultant molecule secreted into the media.

Colony-stimulating factor; Baculovirus; Gene expression; (*Spodoptera frugiperda*)

1. INTRODUCTION

Colony-stimulating factors (CSFs) are a group of glycoproteins required for the survival, proliferation and differentiation of hematopoietic cells [1,2]. One of these factors, hGM-CSF, stimulates the growth of granulocytes and macrophages. hGM-CSF has been expressed in transfected COS cells [3,4] yeast [5,6] and *E. coli* [7] by recombinant DNA techniques. The *E. coli*, yeast, and COS cell systems have disadvantages. hGM-CSF is expressed only transiently and at a relatively low level in transfected COS cells. Yeast-derived hGM-CSF is produced at somewhat higher levels, but can be derivatized with yeast-specific carbohydrate moieties. Expression in *E. coli* has other drawbacks, since *E. coli* neither synthesizes glycoproteins nor secretes 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 a very useful help-independent eucaryotic expression vector for the expression of a wide variety of heterologous genes in large amounts [8]. A late gene product of AcNPV (polyhedrin [9]) can account for up to 50% of the total 'Coomassie stainable' protein of the cell detected on SDS-polyacrylamide gels during a lytic infection. Because this gene is nonessential for replication or production of extracellular viruses in cultured cells, expression vectors that use the strong AcNPV polyhedrin promoter to drive foreign genes have been developed [9]. By using this approach, large amounts of eucaryotic proteins have been successfully produced, including human fibroblast interferon [9], human c-*myc* protein [10] and human interleukin-2 [11]. In addition, insect cells can perform many of the higher eucaryotic post-translational modifications, including glycosylation [9], extracellular secretion [9,11], accurate peptide cleavage [9,11], and phosphorylation [10]. These features of the baculovirus system make it attractive for the large-scale production of recombinant hGM-CSF. In this article, we describe the expression of hGM-CSF in Sf9 cells using the Baculovirus Expression Vector System.

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Abbreviations: hGM-CSF, human granulocyte-macrophage colony-stimulating factor; Sf9, *Spodoptera frugiperda;* AcNPV, *Autographa californica* nuclear polyhedrosis virus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel; hpi, hour postinfection

2. MATERIALS AND METHODS

2.1. *Cells and viruses*

Sf9 cells (ATCC, CRL1711) were grown at 27°C in TNM-FH (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Difco, Detroit, MI). The E2 strain of wild-type AcNPV and pAc373 were kindly provided by Dr Max Summers (Texas A & M University). Procedures for the use of AcNPV as an expression vector have been described in detail [12].

2.2. *Construction of recombinant viruses*

The hGM-CSF sequence in the plasmid p91023(B) (ATCC, 39754) was transferred into the wild-type AcNPV genome by the following procedure. 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.

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This segment was transferred into the baculovirus polyhedrin plasmid pAc373 [11] which had been digested with *Bam*HI and treated with bacterial alkaline phosphatase. A new construct (pAc373GM-CSF) is obtained, containing the intact coding sequence for GM-CSF in the proper orientation downstream of the strong polyhedrin promoter. pAc373GM-CSF DNA (2 μg) was subsequently co-transfected with wild-type AcNPV total viral DNA (1 μg) into SF9 cells using calcium phosphate [13]. After 5 days postinfection at 27°C, a recombinant baculovirus was identified and purified by plaque hybridization [12].

2.3. *Production of hGM-CSF in Sf9 cells*

The time course of hGM-CSF production in vAc373GM-CSF-infected cells was examined in order to detect the optimum time for hGM-CSF expression in the medium. For this, cells (2×10^7) were seeded in 100 mm culture flasks and infected with wild-type or recombinant viruses for 1 h at 27°C. 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 were assayed for biological activity at 24, 48, and 72 h postinfection (hpi). The presence of hGM-CSF-specific polypeptides was also verified by Western blot analysis.

2.4. *Biological assay of hGM-CSF*

hGM-CSF activity assay was performed with either human cord blood cells [14,15] or human bone marrow cells [16]. Approximately 2×10^5 nonadherent mononuclear cells from cord blood or bone marrow was dispensed into each 35 mm Petri dishes containing the test sample. Colonies were counted after 10–12 days. Aggregates of 50 or more cells were scored as a colony using a dissecting microscope.

2.5. *Analysis of proteins by SDS-PAGE and Western blot*

After vAc373GM-CSF infection, conditioned media were prepared and hGM-CSF was analyzed by SDS-PAGE with the discontinuous system of Laemmli [17] and then electrophoretically transferred to nitrocellulose for immunoblot analysis [18]. The nitrocellulose filter was probed with polyclonal rabbit anti-hGM-CSF (Genzyme, Boston, MA) and then developed with goat anti-rabbit antibody-horseradish peroxidase conjugate. For analysis of hGM-CSF activity, 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 mass species (<14 kDa) to 27 kDa, resulting in gel slices 0.25 cm in length and 0.8 cm in width. Each slice was then extracted and assayed for hGM-CSF activity.

2.6. *Glycosylation analysis of expressed hGM-CSF product*

Sf9 cells infected with vAc373GM-CSF at 14 hpi were treated with 5 μg tunicamycin/ml. After 3 days infection, the conditioned medium was collected and analyzed by Western blot. Further, to determine directly if hGM-CSF undergoes *N*-glycosylation in insect cells, protein samples from media of infected Sf9 cells were adjusted to 0.5% SDS and 0.1 M β-mercaptoethanol and boiled for 2 min. The disrupted sample was diluted 3-fold with 200 mM sodium phosphate (pH 8.5). *N*-Glycanase (Genzyme, Boston, MA) was added to a final concentration of 10 U/ml. After incubation for 16 h at 37°C, the sample was analyzed by Western blot.

3. RESULTS

3.1. *Construction and isolation of recombinant viruses*

As shown in fig.1, a segment of hGM-CSF DNA was inserted into the pAc373 transfer vector. A plasmid pAc373GM-CSF was co-transfected into Sf9 cells with wild-type viral DNA. The isolation of the recombinant viruses was further demonstrated by their ability to produce and secrete hGM-CSF into the medium.

3.2. *Production of hGM-CSF activity in Sf9 cells*

Conditioned medium from insect cells infected with

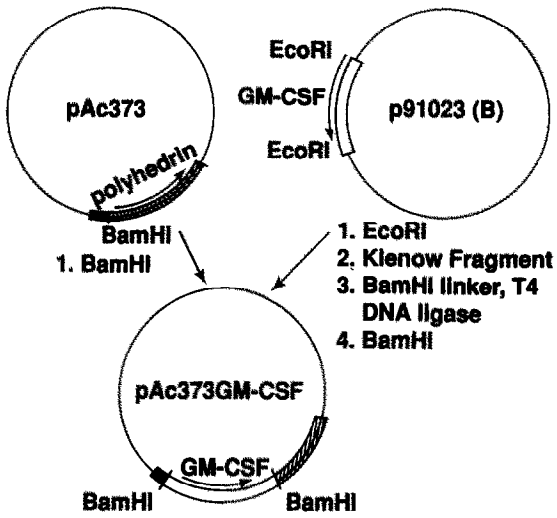

Fig.1. Construction of recombinant transfer vector pAc373GM-CSF. The *Eco*RI fragment of hGM-CSFwas filled in with Klenow fragment. *Bam*HI linkers were added with T4 DNA ligase. The fragment was treated with *Bam*HI and inserted into the unique *Bam*HI site of the transfer vector. The resultant plasmid, pAc373GM-CSF, contained the hGM-CSF sequence placed downstream of the strong polyhedrin promoter.

recombinant viruses was assayed for hGM-CSF activity and analyzed for the presence of hGM-CSF protein by Western blot analysis. As shown in fig.2A, 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 $1\text{–}4 \times 10^7$ U/mg [3], 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 from cells infected with AcNPV had no detectable hGM-CSF activity.

3.3. *Identification of hGM-CSF from infected cells*

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.2B). Three proteins reacting with hGM-CSF antibody were detected to have accumulated in the medium by 48 hpi, of molecular masses 14.5, 15.5 and 16.5 kDa. This appears to suggest the expression of 3 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.3). Biological activity was found to be associated with gel slices over the range from 14 to 17 kDa, suggesting that 3 species of hGM-CSF are biologically active.

3.4. *Glycosylation analysis of recombinant hGM-CSF*

Tunicamycin, an inhibitor of all *N*-glycosylation in glycoprotein biosynthesis [19], was used to determine the nature of glycosylation and its effect on the size of

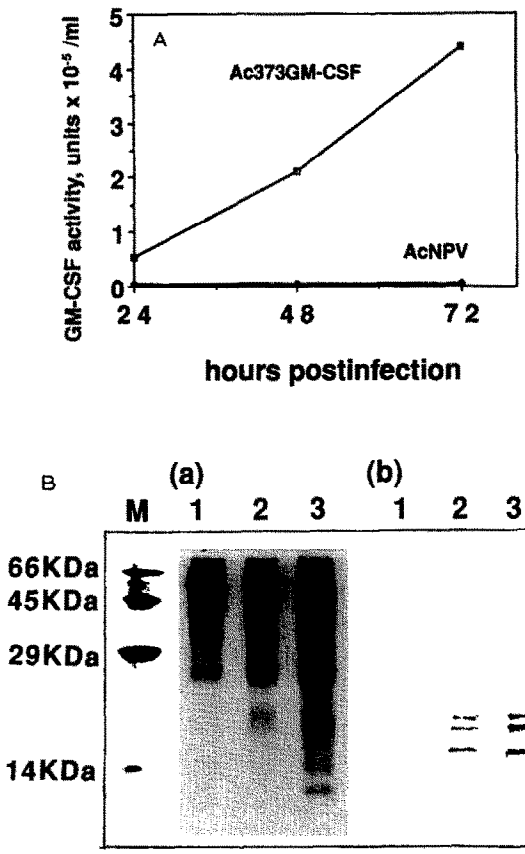

Fig.2. Expression of hGM-CSF in Ac373 GM-CSF-infected Sf9 cells. Cells were infected with vAc373GM-CSF at a cell density of 10^6 cells/ml. At 24, 48 and 72 h postinfection, samples were taken and (A) assayed for hGM-CSF activity as described in section 2. The hGM-CSF activity vAc373GM-CSF-(□) and AcNPV-(♦) infected (B) electrophoresed on a 12% SDS-PAGE gel and stained with Coomassie blue or 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 a set of protein standards.

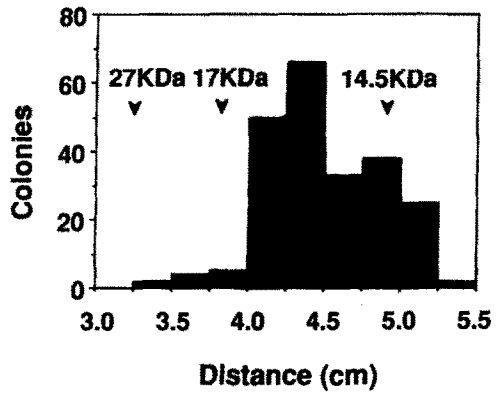

Fig.3. hGM-CSF activity profile on SDS-PAGE. Conditioned medium from vAc373GM-CSF-infected cells was analyzed on a 12% SDS-PAGE gel. The gel was sliced and assayed for hGM-CSF activity as described in section 2.

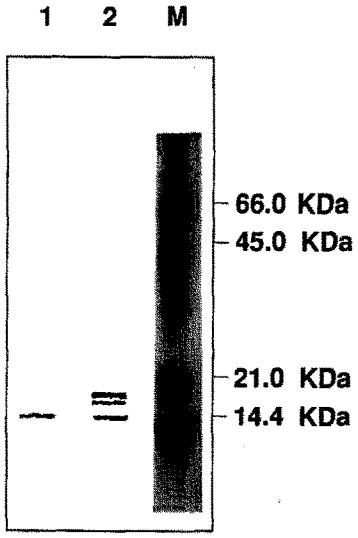

Fig.4. Effect of tunicamycin (TM) on hGM-CSF secretion from Sf9 cells. vAc373GM-CSF-infected Sf9 cells were treated with 5 μg/ml tunicamycin at 14 hpi. At 72 hpi, samples were electrophoresed on a 12% SDS-PAGE gel and electrophoretically transferred to nitrocellulose for immunoblot analysis. (Lanes 1 and 2) Western blot analysis prepared from proteins with and without TM treatment, respectively. (Lane M) Molecular weight markers.

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 aglycoprotein was shown by Western analysis. However, tunicamycin had little effect on the production of the 14.5 kDa hGM-CSF protein in vAc373GM-CSF-infected cells (fig.4). Furthermore, when the hGM-CSF from conditioned medium was treated with *N*-

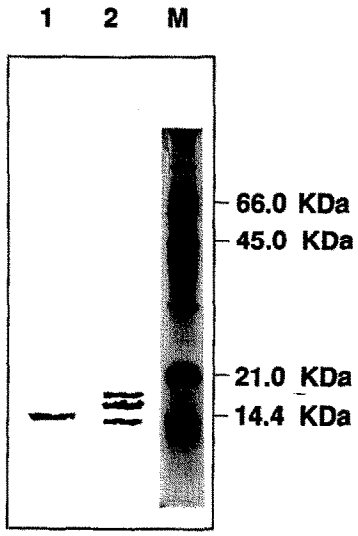

Fig.5. *N*-Glycanase analysis of insect cell-derived hGM-CSF. The protein samples were treated with *N*-glycanase as described in section 2. The reaction products were analyzed by Western blot. (Lanes 1 and 2) Western blot analysis prepared from proteins with and without *N*-glycanase treatment, respectively. (Lane M) Molecular weight markers.

glycanase, the 15.5 and 16.5 kDa of hGM-CSF was converted to a single species with a molecular mass of 14.5–15.5 kDa (fig.5). 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.

4. DISCUSSION

In this study, we used the Baculovirus Expression Vector System (BEVS) for the production of hGM-CSF in insect cells. The hGM-CSF produced in Sf9 cells is capable of stimulating colony formation in the cord blood cell assay. This suggests that the pre-hGM-CSF signal peptide was recognized and cleaved within Sf9 cells and that the hGM-CSF is secreted into the medium during infection.

Western blot analysis revealed the hGM-CSF produced in the culture medium to have apparent molecular masses of 14.5, 15.5 and 16.5 kDa. The two larger bands result from glycosylation heterogeneity and the small one is an unglycosylated form as evidenced by the fact that a single band appears when these glycosylated proteins were treated with *N*-glycanase, or when infected Sf9 cells were treated with tunicamycin. All 3 different hGM-CSF forms secreted by insect cells show hGM-CSF activity, suggesting that differences exist in the posttranslational processing of the gene product resulting in 3 distinct but biologically active forms of the hGM-CSF. Multiple glycosylation products of expressed hGM-CSF [20], haemagglutinin [21] and mouse IL-3 [22] have also been observed in this system.

Many other mammalian glycoproteins expressed in insect cells, for example the human β-interferon [9], human epidermal growth factor receptor [23] and haemagglutinin or influenza virus [21], 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 [24]. Instead, a much shorter and simpler oligosaccharide consisting of (Asn)-$GlcNAc_2$-Man_3 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 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 mass 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 [3] 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.

Our results demonstrate that hGM-CSF protein is expressed at a level of 11–45 mg/l by infected Sf9 cells. Although such expression is lower than that of the viral protein of polyhedrin (1200 mg/l), it is comparable to the expression of other recombinant proteins in this system such as c-*myc* [10], EGF receptor [23] and interleukin-2 [11]. At this level of expression an adequate quantity of functional hGM-CSF protein can be obtained for further physical-chemical analysis.

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