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Expression of Human Thrombopoietin in Insect Cells by Polyhedrin-gp64 Dual Promoter-Based Baculovirus Vector System

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Abstract A new baculovirus transfer vector (pPGP404) was constructed to increase the expression level of human thrombopoietin (hTPO) in insect cells. In pPGP404, hTPO was cloned next to the AcNPV polyhedrin-gp64 dual promoter and the leader sequence of hTPO was substituted with that of gp64. A recombinant baculovirus, AcPGP404, was constructed by using pPGP404 as a transfer vector. hTPO was expressed in AcPGP404-infected TN5 cells and it was observed that the expression levels of hTPO in TN5 cells increased three-fold (6.0 $\mu g/ml^{-1}$) compared to the level expressed under the control of the polyhedrin single promoter. These results indicate that the polyhedrin-gp64 dual promoter system would be useful for expression in large quantities of recombinant proteins in insect cells.

Key words: TPO, dual promoter, gp64, polyhedrin, signal sequence

The baculovirus expression system has become a useful tool for high yield production of functionally active recombinant proteins in insect cells [11]. To date, the very late polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been used for the expression of recombinant proteins in insect cells in most cases. The strong polyhedrin promoter and the insect cells' ability to perform eukaryotic post-translational modifications have made the system useful for the expression of mammalian genes in insect cells.

In spite of these advantages, there are several drawbacks in the baculovirus expression system. The expression levels of glycosylated and secreted foreign proteins are often much lower than those for the recombinant proteins located within the cytoplasm [9]. Proteins expressed at an early phase of infection (24 h post-infection, or hpi) are efficiently glycosylated and secreted to the culture media. However, the levels of glycosylation and secretion of the proteins expressed at very late phases of the infection (after 48 hpi), when the polyhedrin promoter is in the most active state [1, 4], are low. Viral infection may interfere with the cell's ability to process the proteins expressed at very late infection phases and the signal peptide for processing these proteins may not be recognized efficiently by the host cell [10, 12].

One way to overcome these frequently encountered drawbacks in baculovirus expression systems would be substitution of the native signal peptide with that of either honeybee prepromellitin [13] or gp64 of AcNPV [10, 15].

Human thrombopoietin (hTPO) is a hematopoietic growth factor that specifically stimulates the proliferation and maturation of megakaryocytes, leading to the production of platelets [3, 8]. Although biologically active hTPO can be expressed in insect cells using the baculovirus expression system [7], improvement of the expression level is still required for mass production.

The purpose of this study was to improve the expression levels of secreted hTPO by using the novel polyhedringp-64 dual promoter of AcNPV in insect cells. The effects of the substitution of the signal peptide of native hTPO with that of AcNPV gp64 was also investigated. The expression levels of hTPO in insect cells under the control of the polyhedrin-gp64 dual promoter increased three-fold compared to that expressed under the control of the polyhedrin single promoter.

Materials and Methods

Cell Lines

The Sf9 subclone of IPLB-SF21-AE and TN5 cells (BTI-TN-5B1-4) derived from *Trichoplusia ni* egg cell homogenates (Invitrogen, Carlshad, U.S.A.) were cultured at 27°C in SF900II serum free media (GIBCO-BRL, Gaithersburg, U.S.A.) as a monolayer.

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Construction of New Baculovirus Transfer Vectors

The full-length hTPO (353 amino acids) cDNA containing a native signal sequence was amplified by polymerase chain reaction (PCR) using pBlue404 as a template [7]. The sense primer (404-*Bgl*II), containing the *Bgl*II site was 5'-GAAGATCTATGGAGCTGACTGAA-3' and the antisense primer (404-*Eco*RI) containing the *Eco*RI site was 5'-ATGAATTCTCACCCTTCCCTGAGAC-3'. The *Bgl*II/*Eco*RI fragment of hTPO cDNA (1.0 kbp) was subcloned into the corresponding sites of the pBlueBac4 (Invitrogen) and named pBT332 [Fig. 1(a)]. To substitute the signal sequence of hTPO with that of AcNPV gp64, the signal sequence of gp64 was amplified by PCR using pGP64 as a template [6]. The 5' sense primer (64-A) containing the *Bgl*II site and the N-terminal 3 amino acid codons of the gp64 signal peptide was 5'-GGAGATCTATGGTAAGCGC-3'. The 3' antisense primer (64-B) containing the *Bgl*I site and the last 5 codons of the gp64 signal sequence was 5'-CCGCCGCAAAGGCAGAAT-3'. The *Bgl*II/*Bgl*I fragment (63 bp) of the amplified PCR product was ligated to the *Bgl*I/*Eco*RI PCR fragment (1005 bp) encoding mature hTPO cDNA. The ligated *Bgl*II/*Eco*RI fragment (1068 bp) was cloned into the *Bgl*II/*Eco*RI sites of the pBT332, and named pGP404 [Fig. 1(b)]. The promoter region (292 bp) and signal sequence of AcNPV gp64 was amplified by PCR using pGP64 as a template. The upstream primer (64-pro) containing a *Bgl*II site and upstream promoter region (-292 bp from the translation start codon of gp64) was 5'-GGAGATCTTAGCCATCGTGATCGC-3'. The downstream primer was the one used for amplification of the signal

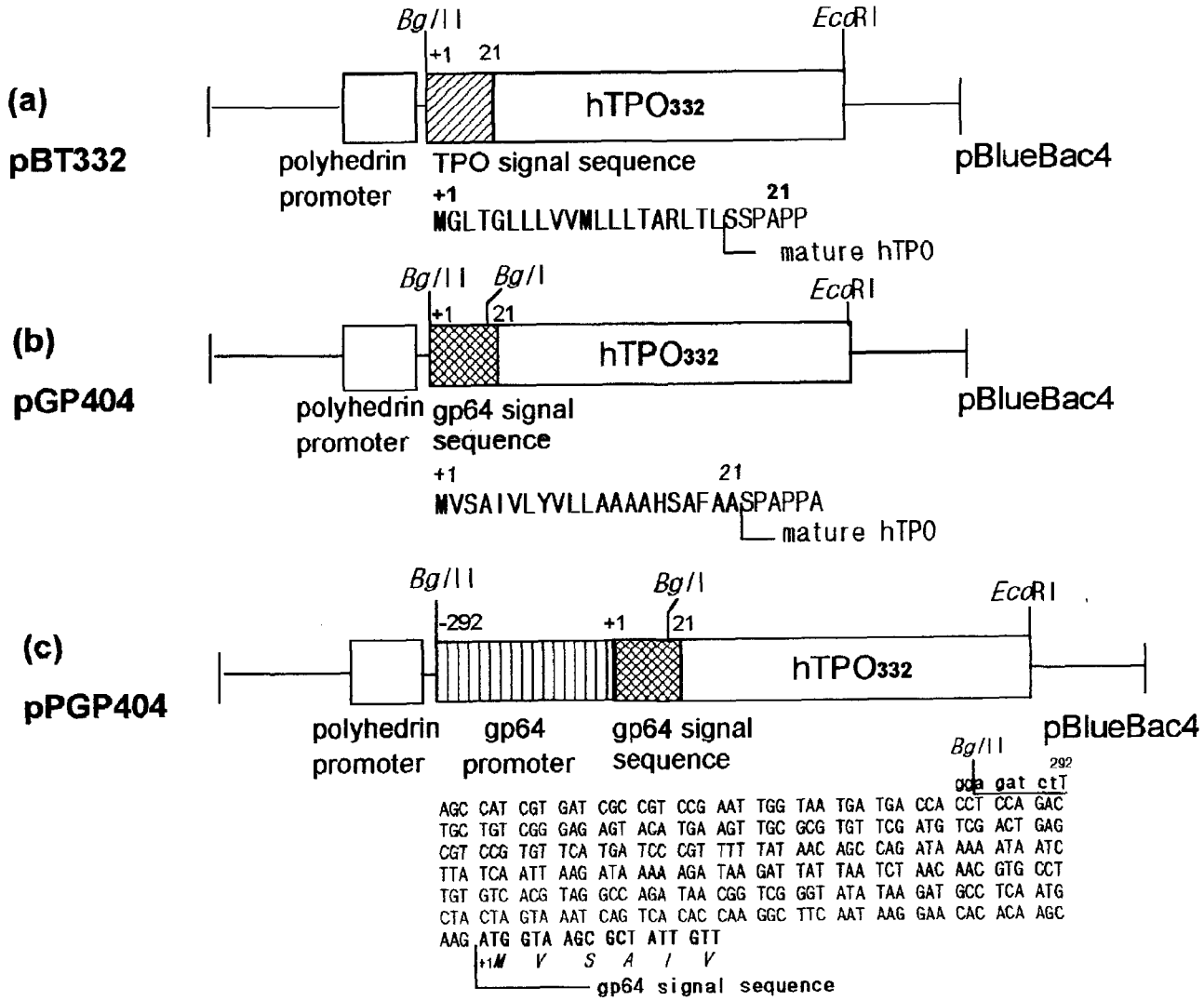

Fig. 1. Schematic diagram of baculovirus transfer vectors, pBT332, pGP404, and pPGP404.
(a) pBT332 was constructed by inserting full-length hTPO cDNA next to the polyhedrin promoter in pBlueBac4. (b) pGP404 was generated from pBT322 by replacing hTPO signal sequence with that of gp64. (c) pPGP404 was constructed by inserting early and late promoters of gp64 next to the polyhedrin promoter of pGP404.

sequence of gp64 (64-B). A 355 bp of a *Bgl*II/*Bgl*I fragment of the PCR product was ligated to the *Bgl*II/*Eco*RI PCR fragment (1,005 bp) encoding mature hTPO cDNA. The ligated *Bgl*II/*Eco*RI fragment (1,360 bp) was cloned into the *Bgl*II/*Eco*RI sites of the pBT332, and named pPGP404 [Fig. 1(c)].

Construction of Recombinant Viruses and Expression of hTPO in Insect Cells

pBT332, pGP404, and pPGP404 DNAs and triple-cut linear AcNPV Bac-N-Blue DNA (Invitrogen) were co-transfected into Sf9 cells by InsectinPlus liposomes (Invitrogen). Recombinant viruses isolated from the blue plaques were further purified by three rounds of plaque assay and named Ac332, AcGP404, and AcPGP404, respectively. TN5 cells grown in Sf900II serum-free medium were infected with recombinant virus at a multiplicity of infection (MOI) of 10. At various time intervals, 1 ml aliquots were removed from the culture vessel and the cells were harvested by centrifugation at 13,000 rpm for 2 min. Both the cells and the tissue culture media were collected for analysis.

ELISA and Western Blot Analysis

The amount of hTPO was measured by antigen-capture ELISA. The purified recombinant hTPO (R&D Systems, McKinley, U.S.A.) was used as a standard. The serially diluted proteins were added into a 96-well plate and incubated at 4°C for 18 h. After blocking the plate with 5% bovine serum albumin in Dulbecco's phosphate buffered saline (PBS), 100 µl of goat anti-human polyclonal antibody (1:1000 dilution, R&D Systems) was added and incubated at 37°C for 2 h. After washing the plate with PBS, bound IgG was detected by alkaline phosphatase-conjugated goat anti-human IgG (1:10,000 dilution, Sigma, St. Louis, U.S.A.). Then, 100 µl of *p*-nitrophenyl phosphate substrate solution (Sigma) was added and incubated for 30 min. The optical density was measured at 405 nm on a microplate reader. For Western blot analysis, an equal volume (10 µl) of the medium fraction was resolved by electrophoresis on a 10- 15% gradient polyacrylamide gel. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane in a buffer containing 92 mM glycine, 25 mM Tris, and 20% (v/v) methanol (pH 8.5). Blots were blocked with 5% non-fat dry milk. Then, 1:1000 dilution of the goat antibody against hTPO (R&D Systems) in TBS (50 mM Tris-HCl, pH 7.5, 45 mM NaCl, and 0.1% Tween 20) was added and incubated at room temperature for 2 h. After three washes with TBS, a 1:10,000 dilution of the alkaline phosphatase-conjugated anti-goat IgG (Sigma) in TBS was added and incubated for an additional 2 h under the same conditions. Development of the color was performed with NBT and BCIP as described in the manufacturer's protocol (Sigma).

Results and Discussion

Although hTPO produced in insect cells by the baculovirus expression system was glycosylated and found to be biologically active [7], improvement of the expression level is required for mass production of hTPO for future studies. The object of this study was to increase the expression level of the recombinant protein in insect cells by using the AcNPV polyhedrin-gp64 dual promoter.

The gp64 promoter is active in the early and late phases of virus infection [2, 5], while the polyhedrin promoter is specific at a very late phase of the viral infection [14]. By using the dual promoter, consisting of gp64 and polyhedrin, we attempted to extend the expression period from early to very late phases of the virus replication.

The pGP404 was constructed from pBT322 by substituting the signal sequence of hTPO with that of gp64. The pBT332 was constructed by cloning hTPO cDNA carrying the native signal sequence next to the polyhedrin promoter of the pBlueBac4. The pPGP404 was generated from pGP404 by inserting the early and late promoters of gp64 next to the very late polyhedrin promoter.

Each transfer vector plasmid and linear AcNPV DNA were co-transfected into Sf9 cells to construct recombinant AcNPVs by cell-mediated homologous recombination. Recombinant baculoviruses derived from pBT332, pGP404, and pPGP404, were isolated, plaque purified, and named Ac332, AcGP404, and AcPGP404, respectively. The presence of hTPO cDNA in the recombinant viruses was confirmed by PCR (data not shown).

TN5 cells grown in a T-175 flask to a density of 1×10^7 cells were seeded to 1×10^6 cells/well in 6-well plates. The cells were infected with each recombinant virus at 10 MOI and the levels of hTPO in the culture media collected at various post-infection time intervals were analyzed by ELISA.

The amounts of hTPO in the culture media at 24 and 48 hpi were slightly higher in TN5 cells infected by AcGP404 compared to those infected by Ac322. This result indicated that replacement of the native leader peptide of hTPO with that of AcNPV gp64 enhanced secretion of the hTPO into the culture media (Fig. 2). Of the three recombinant viruses, AcPGP404 showed the highest expression level (about 6.0 µg/ml), which is about three times higher than those of Ac332 and AcGP404 (Fig. 2). The amount of hTPO expressed and secreted into the culture medium reached a maximum at 48 hpi and persisted until 72 hpi (Fig. 2). Since expression of the hTPO in AcPGP404-infected TN5 cells would be controlled by the polyhedrin-gp64 dual promoter, these results suggest that the polyhedrin-gp64 dual promoter was stronger than was the polyhedrin single promoter alone.

The recombinant hTPO expressed in TN5 cells was also analyzed by Western blot. The quantity of hTPO measured

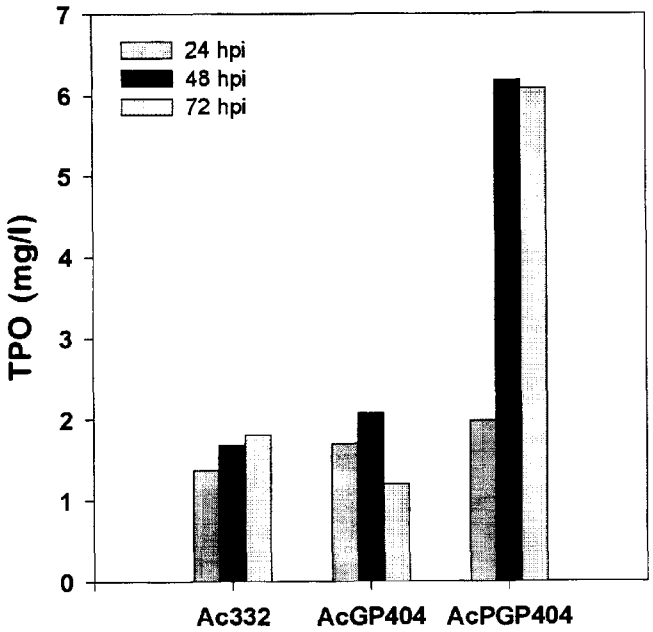

Fig. 2. Time course expression of hTPO in insect cells infected with recombinant viruses.
Monolayers of TN5 cells cultured in 6-well plates (1×10^6 cells/well) were infected with recombinant viruses Ac332, AcGP404, and AcPGP404 at 10 MOI. Culture media was collected at each time course interval and the amount of expressed hTPO was analyzed by ELISA.

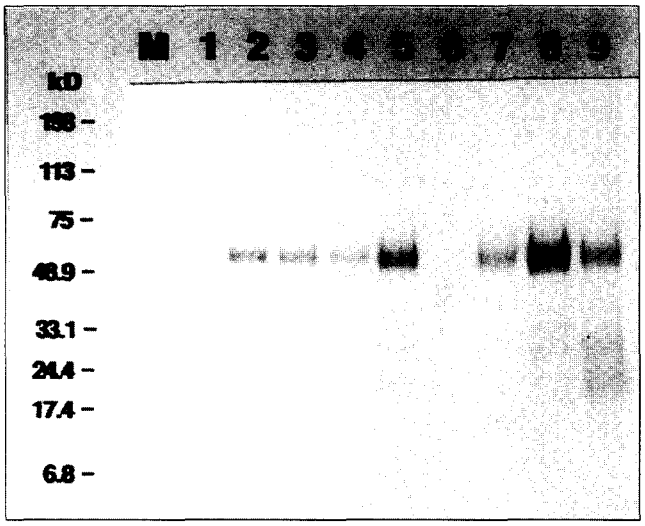

Fig. 3. Western blot analysis of the recombinant hTPO expressed in TN5 cells.
Monolayers of TN5 cells cultured in 6-well plates (1×10^6 cells/well) were infected with Ac332, AcGP404, and AcPGP404 at 10 MOI. Culture medium was harvested at each time course interval. 10 μl of each sample was run on a 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoblot analysis was performed as described in Materials and Methods. Lanes 1, 4, and 7, 24 hpi; lanes 2, 5, and 8, 48 hpi; lanes 3, 6, and 9, 72 hpi. Lane M, pre-stained molecular weight marker (Bio-Rad).

by ELISA corresponded with the intensity of the protein bands shown in Western blot analysis (Fig. 3). Expression levels of hTPO in AcPGP404-infected TN5 cells (Fig. 3, lane 8) were higher than in AcGP404-infected TN5 cells (Fig. 3, lane 5), suggesting that the polyhedrin-gp64 dual promoter was stronger than the polyhedrin single promoter alone. These results imply that under the dual promoter system, the early and late promoters of gp64, which have maximum effect at 24-h post-infection, and the polyhedrin promoter, which is in an active state at 48-h post-infection, could act sequentially in infected insect cells.

hTPO carrying the signal peptide of gp64 was detected at 24 hpi (Fig. 3, lane 4), while hTPO with native signal peptide was detected at 48 hpi (Fig. 3, lane 1). These results suggest that the signal sequence of gp64 was efficiently recognized by the insect cell secretion system compared to that of hTPO. The band intensity of hTPO collected at 72 hpi was weaker than that of hTPO collected at 48 hpi, and several smaller size bands were detected at 72 hpi (Fig. 3, lanes 8 and 9). The smaller bands observed at 72 hpi are most likely derived from the degradation of intact hTPO by host protease.

The results obtained from this study demonstrate that the polyhedrin-gp64 dual promoter system would be useful for production of large amounts of eukaryotic proteins in insect cells.

Acknowledgments

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