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Temporal nature of the promoter and not relative strength determines the expression of an extensively processed protein in a baculovirus system

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We demonstrate that the expression of extensively modified and secreted heterologous proteins synthesized in the baculovirus expression vector system (BEVS) depends on the temporal nature of the promoter transcribing the foreign gene. The β subunit of the human chorionic gonadotropin, an extensively modified secretory glycoprotein hormone was expressed under the transcriptional control of the AcNPV basic protein gene promoter (MP) and the polyhedrin gene promoter (POL), respectively. MP, activated late in the infection cycle, is a weaker promoter when compared to the stronger very late POL promoter. Levels of secretion, immunoreactivity and bioactivity of recombinant proteins, βhCG$_{MP}$ and βhCG$_{POL}$ synthesized under control of the MP and POL promoter were compared. Secretion of βhCG$_{MP}$ was relatively higher. Enzymatic analysis revealed that the synthesized protein was sialylated. Receptor binding assays and testosterone stimulation assays in a mouse Leydig cell system demonstrated that on a unit protein basis, βhCG$_{MP}$ was biologically more active than βhCG$_{POL}$.

Temporal promoter; Secretion; Biological activity; Sialylation; Insect cell

1. INTRODUCTION

The baculovirus expression vector system (BEVS) uses a helper-independent virus that can hypertranscribe and express to very high levels, in lepidopteran cell lines and caterpillars, a foreign gene placed under the control of the very late polyhedrin gene promoter. BEVS is very widely used since the recombinant protein synthesized in this system is similar to its authentic counterpart in terms of immunoreactivity, immunogenicity and bioactivity [1]. However, it has been commonly observed that membrane-associated, secretory or heavily glycosylated proteins are expressed in relatively lower amounts. HBsAg, for instance is only partially secreted to a level of 0.3 mg/l at five days post-infection (p.i.) [2]. Human multidrug transporter [3] and the chorionic gonadotropin α subunit [4] which are secretory glycoproteins as well as preproattacin [5] have been shown to be inefficiently secreted. On the other hand, genes encoding non-secretory proteins such as luciferase [6], influenza virus hemagglutinin [7], bovine leukemia virus p34 protein [8], the bluetongue virus NS1 protein [9] etc. are expressed to much higher levels, at times comparable with that of polyhedrin. This phenomenon of bias against secretory proteins is more evident using a recombinant dual expression virus carrying a secretory and non-secretory gene, where expression of the non-secretory protein is severalfold higher than the secretory one, even though the genes encoding them are placed under identical transcriptional control [10]. Therefore, the amount of biologically and functionally active protein synthesized in BEVS is not solely dependent on the strength of the promoter.

We previously reported the expression and bioactivity of the α-subunit of hCG [4]. αhCG, a glycosylated, secretory protein, was shown to be inefficiently secreted with the bulk of the expressed protein remaining behind in the cell pellet as compared to the supernatant. This was because of overexpression of protein which could not be completely processed and secreted within the short time available prior to cell death caused by the lytic nature of the virus. This 'secretory load' [4] is the manifestation of the inability of the cells to cope with the rapidly accumulating mass of foreign protein synthesized under transcriptional control of the hyperactive and temporally regulated very late polyhedrin (p29) gene promoter. The p10 promoter, another strong but very late promoter or the synthetic promoter carrying the consensus sequence of the polyhedrin and p10 pro-

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Abbreviations. AcNPV, *Autographa californica* nuclear polyhedrosis virus; hCG, human chorionic gonadotropin; Sf9, *Spodoptera frugiperda* clone 9 insect cells; βhCG$_{POL}$, secretory recombinant βhCG synthesized under polyhedrin promoter control; βhCG$_{MP}$, secretory recombinant βhCG synthesized under MP1 promoter control; vAcβhCG_{POL}, recombinant baculovirus which expresses the βhCG gene under polyhedrin promoter control; vAcβhCG_{MP}, recombinant baculovirus which expresses the βhCG gene under MP1 promoter control.

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moters used in this system face somewhat similar problems [11–13]. Here, we describe the results of expression of the β subunit of hCG, a heavily glycosylated, secretory and complex protein, under the viral polyhedrin and basic protein (MP) promoters. The MP promoter is weaker than the polyhedrin promoter and peaks in activity at an earlier time p.i. [14], thus giving more time for post-translational modifications between promoter activation and cell lysis. Using βhCG as a reporter, we show that the temporal nature of the activation of the promoter and not its relative strength is responsible for efficient secretion, glycosylation and bioactivity of recombinant βhCG. We also show that insect cells are indeed capable of sialylating the expressed protein.

2. MATERIALS AND METHODS

2.1. *Construction, purification and characterisation of recombinant viruses*

The 539 bp βhCG cDNA was excised from pBS2 [15] as a *Bgl*II fragment. This was placed separately under the transcriptional control of the polyhedrin promoter and the basic protein promoters to construct recombinant transfer vectors pVcβhCG_{POL} and pAcβhCG_{MP}, respectively. The recombinant plasmids were characterized by restriction enzyme digestion and in-gel hybridisation [16] for the presence of the βhCG gene insert in the correct orientation with respect to the corresponding promoter. Recombinant baculoviruses vAcβhCG_{POL} and vAcβhCG_{MP} were isolated, purified and titrated as before [17].

2.2. *Synthesis and bioactivity of hCG*

Identical titres of each recombinant virus was used to infect Sf9 cells seeded in 24-well plate. βhCG expression in the culture supernatant was monitored at 12, 24, 36, 48 and 72 h p.i. by a subunit specific RIA [15]. The amount of hCG as estimated by RIA was normalized to 1 μg in each case and incubated with a large molar excess of purified standard αhCG for 16 h at 37°C. The αhCG/βhCG dimer was quantitated using a rat testicular receptor binding assay [18] and a mouse Leydig cell bioassay [19] with appropriate modifications [17].

2.3. *Purification and analysis of recombinant protein*

Suspension cultures of Sf9 cells were infected at high multiplicity of infection with vAcβhCG_{POL} and vAcβhCG_{MP} and the culture supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C to precipitate the virus particles. An affinity column carrying immobilized monoclonal antibody specific to βhCG was pre-equilibrated with 0.01 M Tris-HCl, pH 8, containing 0.14 M NaCl which was recirculated overnight to which the virus-free culture supernatant containing the secretory, recombinant βhCG was loaded. The column was then washed once with 10 mM Tris-HCl, pH 8 containing 0.14 M NaCl and twice with 50 mM Tris-HCl, pH 8 containing 0.5 M NaCl, 0.1% Triton X-100. The recombinant βhCG protein was eluted with 0.05 M glycine buffer pH 2.3 containing 0.15 M NaCl [20]. Peak fractions were pooled, dialysed and the concentration of the recombinant purified protein determined by RIA. Purified protein was characterised by Western blotting as described [17,21]. Commercially available sialic acid detection (Boehringer Mannheim) was used as per the manufacturer's recommendations for quantitative estimation of sialic acid residues. Urinary βhCG was used as an external control.

3. RESULTS AND DISCUSSION

3.1. *Expression, secretion and bioactivity of βhCG*

Partial restriction maps of pVcβhCG$_{POL}$ and pAcβhCG$_{MP}$ showing the direction of transcription of the βhCG gene under the different promoters are depicted in Fig. 1. Sf9 cells seeded in a 24-well plate were infected with the same titre of vAcβhCG_{POL} and vAcβhCG_{MP} and at different times p.i., the culture supernatant was assayed for the presence of βhCG using RIA. In vAcβhCG_{POL}, the peak of βhCG production followed the kinetics of the polyhedrin promoter activity giving 2.8 μg/2 × 10^6 at 48 h p.i. reaching a maximum level of 9.05 μg/2 × 10^6 cells at 72 h p.i. whereas, in vAcβhCG_{MP} the peak of βhCG secretion reached 11.3 μg/2 × 10^6 cells at 48 h p.i. (Fig. 2A). As would be expected, the levels of secretory protein were relatively higher under the control of the MP promoter at initial time p.i. hCG is a heteromeric protein composed of two subunits, α and β. The individual subunits of hCG on their own have no biological activity and require to associate with the corresponding subunit to give a bio-

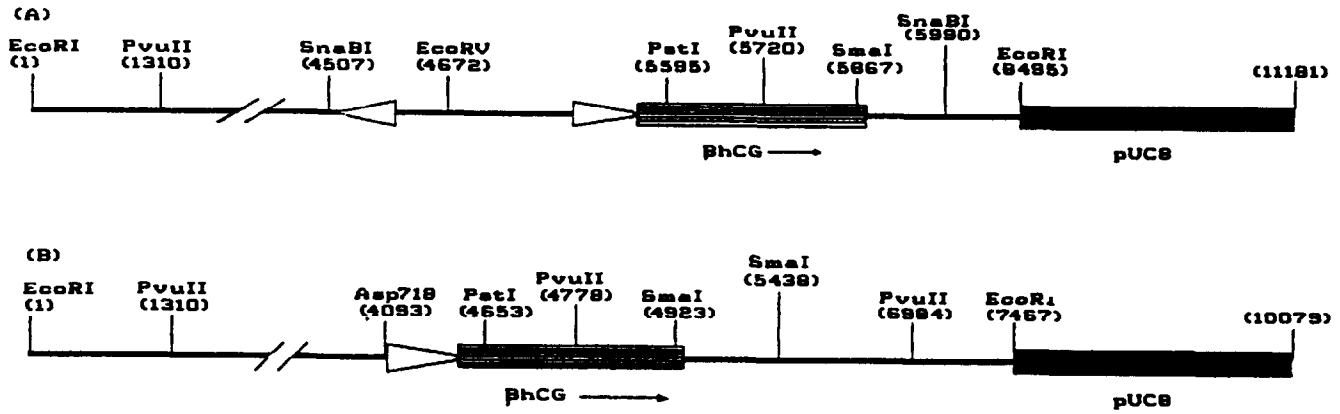

Fig. 1. Partial physical maps of recombinant plasmids pVcβhCG_{POL} (A) and pAcβhCG_{MP} (B). The integration of the βhCG gene under the control of the polyhedrin (POL) promoter and the basic protein (MP) promoter, respectively, is illustrated (not drawn to scale). Those restriction sites used for checking the orientation of the insert with respect to the promoter are indicated. The sequences upstream and downstream of the foreign gene are identical to the polyhedrin gene flanking sequences present in the viral DNA to enable homologous recombination at the polyhedrin locus. The numbers indicate the positions of different restriction enzyme sites in the recombinant transfer vector with respect to the nucleotide 'A' within the 5′ *Eco*RI site of the viral *Eco*RI-I fragment.

Table I
Sialic acid analysis of recombinant βhCG

	% sialic acid
Native βhCG	10
βhCG	6.3
βhCG	8

logically active αβ heterodimer. The recombinant βhCG was checked for its biological activity. Mock-infected cells showed no activity. It is interesting to note (Fig. 2B) that βhCG showed higher levels of the biologically active molecules when compared to βhCG (80% vs. 40%).

3.2. *Purification and analyses of recombinant protein*

Recombinant βhCG was purified from culture supernatant by immunoaffinity using the specific monoclonal antibody to βhCG and characterised on SDS-PAGE. This was further immunoblotted to confirm the authenticity of the eluted sample. The recombinant βhCG produced in insect cells exhibited increased electrophoretic mobility when compared to the native βhCG purified from urine of pregnant women. Native βhCG was detected as a 33 kDa band while the recombinant protein was approximately 23 kDa (Fig. 3). In spite of the difference in mobility, there was no loss of immunoreactivity of the insect-derived βhCG.

The difference in electrophoretic mobility was attributed to differences in the level of glycosylation of the proteins and not to the amino acid backbone per-se since total amino acid analysis of native βhCG, βhCG and βhCG did not show significant differences (data not shown). Digestion with sialidase (neuraminidase) was carried out, using a commercial sialic acid detection kit, to check for the ability of the insect cell system to sialylate. Results (Table I) demonstrated the presence of sialic acid residues on the recombinant Sf9-derived protein and the degree of sialylation between the two recombinant proteins (βhCG, βhCG) was quantitatively different.

We have used two different promoters to direct the transcription of the βhCG gene and evaluated the expression of the β subunit of hCG in insect cells using BEVS. The very commonly used AcNPV polyhedrin gene promoter is activated very late in the viral life cycle and soon after the occlusion bodies are formed, cell lysis occurs due to the lytic nature of the baculovirus. Polyhedrin, being a non-secretory, non-glycosylated protein, is expressed to very high levels to occlude the viral particles, following which cell death occurs. It has already been shown previously [2,3,6,8,9] that expression under polyhedrin promoter control varies considerably, which could be attributed to various factors. Presence of specific sequences around the ATG [22], secondary

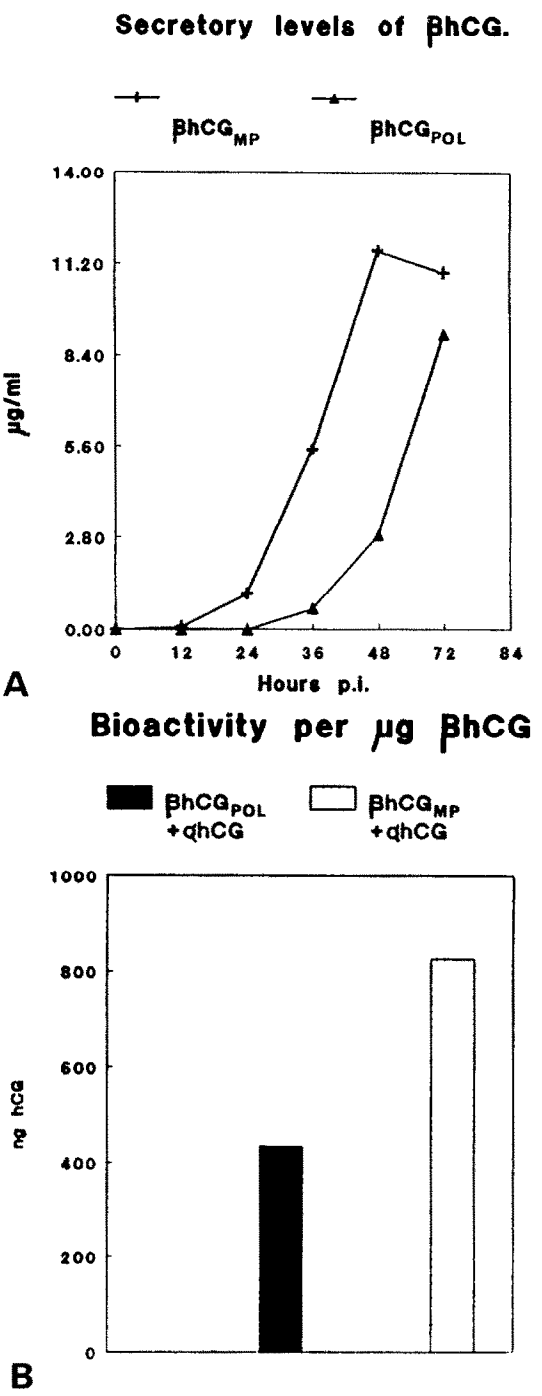

Fig. 2. Time course and biological activity of βhCG synthesized in insect cells infected with vAc*βhCG* and vAc*βhCG*. (A) Recombinant baculoviruses vAc*βhCG* and vAc*βhCG* were isolated, purified and titrated. Identical titres of each recombinant virus were used to infect Sf9 cells seeded in 24-well plate. βhCG expression in the culture supernatant was monitored at 12, 24, 36, 48 and 72 h p.i. by a subunit-specific RIA. (B) Biological activity of βhCG and βhCG. Supernatant from infected cells was incubated with a molar excess of purified standard αhCG for 16 h at 37°C and the αhCG/βhCG dimer was quantitated using a Leydig cell bioassay. These results are the average of at least five experiments and variations between experiments were less than 10 ng.

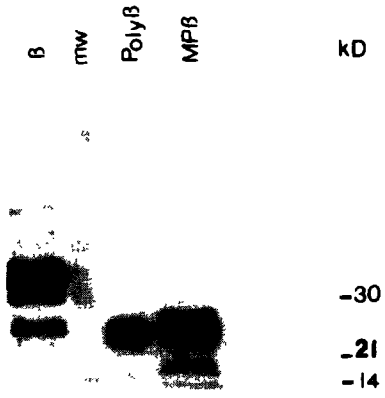

Fig. 3. SDS-PAGE of affinity purified βhCG$_{MP}$, βhCG$_{POL}$ and native, urinary βhCG.

structure of the foreign gene transcript and mRNA stability, amongst others, could be responsible. In BEVS, the cellular machinery has an important role to play in carrying out the various post-translational modifications. An intact, functional system is required if an efficiently processed product is to be synthesized which will not be possible if cell lysis occurs soon after the production of the recombinant protein. In a recent study [23], a time course of HIV envelope protein expression revealed that at early times p.i. (24 h) the proteins were fully glycosylated and soluble in non-ionic detergents. However, at 48 h p.i. and onwards, expression levels of the recombinant protein reached a maximum but most of the increase was due to a rise in the level of non-glycosylated species which were largely insoluble. Thus, it appeared that Sf9 cells could not process large amounts of recombinant products very efficiently. Another recent study [24] demonstrates that lepidopteran cells require long periods of time p.i. with a recombinant virus to carry out complex oligosaccharide processing as shown using human plasminogen as a reporter. At early times p.i., there is a preponderance of high-mannose type oligosaccharides on the protein, whereas at late times p.i. (60–96 h) most of the oligosaccharides exist as complex carbohydrates.

We worked on the assumption that an extensively processed protein can be efficiently expressed and secreted to high levels not by using a strong promoter, but by advancing the activation of transcription of the gene such that the cells are given enough time between the peak of protein expression and cell death. βhCG was chosen as a model because it provides a typical example of a protein where extensive post-translational modifications are necessary for biological activity. βhCG is extensively glycosylated (33–36%) with four *O*-linked and two *N*-linked groups [20], besides the presence of sialic acid residues. The sugars play an important role in secretion, subunit assembly, biological activity and stability of hCG [25]. The molecule has six intrachain disulphide bonds which are very important for the native conformation of the molecule. Reduction of these bonds leads to a loss of receptor recognition. This very complex nature of βhCG protein involving extensive post-translational modifications, processing and proper folding makes it an excellent reporter for our study. The gene encoding βhCG, was separately cloned under the control of both the polyhedrin promoter as well as the basic protein promoter MP. The basic protein is an essential structural protein of the virus which is expressed earlier in the viral life cycle than polyhedrin [14]. Relatively early transcription from the MP promoter (12–24 h) compared to the polyhedrin promoter (24–72 h) has also been demonstrated using the β-galactosidase protein as a reporter where it was also shown that MP is a significantly weaker promoter in terms of its transcriptional activity when compared to the very late polyhedrin gene promoter [14]. This therefore provides a good system to directly measure the importance of promoter strength versus time of promoter activation for the efficient synthesis, secretion and bioactivity of a complex protein like βhCG.

The MP promoter allows the cells a longer time period between the peak of expression and cell lysis during which time the processing pathways do not get compromised which otherwise happens when the foreign gene is expressed under control of the polyhedrin promoter. Data in Figs. 2 and 3 show that immunologically active βhCG$_{MP}$ is more efficiently secreted than βhCG$_{POL}$ in spite of the fact that the former is the result of transcription from a comparatively weaker promoter. The increased time available, when transcribed relatively early in the infection cycle under MP promoter control, allows proper processing and extracellular transport. In a parallel situation, we recently showed [17] that the extended time available to *Spodoptera* larvae upon infection with a recombinant baculovirus results in hCG of a better quality in terms of bioactivity. It was generally believed that lepidopteran cells are unable to perform sialylation. Sialic acids, however, play an important role in a number of biological processes and their expression has been very recently correlated with developmental regulation and early embryogenesis in *D. melanogaster* [26]. Our observation that the baculovirus system cannot only sialylate but that the degree of sialylation can also be manipulated, should enable a more critical evaluation of the role of sialic acid in cellular processes. βhCG$_{MP}$ has a higher percentage of biologically active molecules per unit mass of protein than βhCG$_{POL}$ and this correlates very well with the difference observed in the level of glycosylation (data not shown) between the two recombinant proteins since it is well documented that glycosylation is indeed important for signal transduction leading to steroidogenesis [27,28]. Perhaps, carbohydrates favour proper folding of the subunit resulting in a molecule which can more efficiently interact with the cellular receptors leading to

more efficient transduction of signal(s) for testosterone production. It should be possible now to address specific questions about the role of carbohydrates in folding/bioactivity of hCG or similar molecules.

The present work clearly demonstrates that the temporal nature of a promoter is a more important characteristic than relative strength for the expression of a gene. This has important implications in using the MP or similar promoters activated earlier in the infection cycle [29] for the expression of highly processed proteins since the use of a strong promoter does not necessarily confer any advantage by virtue of its strength [30] in such situations. This approach of temporal expression can be successfully used to produce more efficiently similar proteins, of biomedical and therapeutic uses, which require extensive post-translational modifications and processing for their bioactivity. The capability of the baculovirus system to sialylate makes it more versatile for expressing proteins where sialylation is necessary.

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