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Simultaneous synthesis of enzymatically active luciferase and biologically active β subunit of human chorionic gonadotropin in caterpillars infected with a recombinant baculovirus

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The β subunit of human chorionic gonadotropin (βhCG), a secretory and extensively glycosylated hormone, and firefly luciferase, a non-secretory enzyme, were simultaneously synthesized in *Spodoptera* larvae upon infection with a dual expression recombinant baculovirus, vAc*βhCG-luc*. Luciferase was retained predominantly in the body tissue while βhCG was secreted into the hemolymph of infected larvae. Both the proteins were similar to their authentic counterparts in terms of immunoreactivity and bioactivity. The caterpillar-derived recombinant hCG exhibited reduced electrophoretic mobility on SDS-PAGE and increased biological activity as compared to the hCG expressed in insect cells in culture. The implications of using the larval system for expressing an extensively glycosylated protein are discussed.

β subunit of human chorionic gonadotropin, βhCG; Baculovirus system; Dual expression; Luciferase; Recombinant DNA; Secretion; Glycosylation

1. INTRODUCTION

Baculovirus expression vector system (BEVS) is commonly used for efficient expression of eukaryotic genes [1-3]. Insect cells provide a suitable environment for post-translational modifications and folding of the protein product [4] such that the foreign proteins synthesized are identical to their authentic counterparts in almost all respects [5]. Simultaneous expression of two or more genes within a single cell traditionally required co-infection with two or more recombinant viruses, each containing a single foreign gene [3,6] and the success of this approach depended upon achievement of efficient infection at high multiplicity of infection (MOI) of each virus [4]. However, to address some of the more challenging aspects of biochemistry and molecular biology, such as simultaneous expression of two or more genes in the same cell, synthesis of products involving multiple proteins or consecutive enzymatic factors, and delineation of the factors involved in protein–protein interactions, new eukaryotic expression systems that produce two or more gene products simultaneously are required [4,7]. In situations where an equivalent stoichiometry of expression of the two proteins is desirable, a double recombinant virus ensures an equal delivery of each transcription unit to every infected cell [2]. Such multiple expression vectors have been constructed and used to simultaneously express two foreign genes [3,7,8].

Established cell lines are available in BEVS from several insects such as *Spodoptera frugiperda*, *Bombyx mori* and *Trichoplusia ni* [1,4,5] which give good yields of the synthesized foreign protein. The insect larval system, however, offers an exciting alternative because of the low costs involved in the mass scale production (rearing and maintenance of larvae) as compared to tissue-cultured cells, and also because several proteins are produced in 50- to 500-fold excess in larvae than is achieved in the cell lines [9]. Furthermore, expression in whole insect larvae may provide specialized cell types (such as secretory cells) and specialized post-translational modification enzymes which may be valuable for the expression of some genes [4]. Here we describe the simultaneous synthesis of two unrelated and differently destined proteins, luciferase and the β subunit of human chorionic gonadotropin (βhCG), in *Spodoptera* larvae infected with a genetically engineered baculovirus carrying the genes encoding the respective proteins.

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Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; BEVS, baculovirus expression vector system; hCG, human chorionic gonadotropin; IgG-HRP, immunoglobulin-horse radish peroxidase; luc, firefly luciferase; MOI, multiplicity of infection; PBS, phosphate-buffered saline; p.i., post infection; Sf9, *Spodoptera frugiperda* clone 9 cells.

2. MATERIALS AND METHODS

2.1. *DNA manipulation and construction of recombinant virus*

Plasmid DNA manipulation, co-transfection, isolation, purification and characterization of the recombinant virus, vAc*βhCG-luc* (AcNPV, carrying the genes encoding βhCG and luciferase (luc) under the control of duplicate copies of the polyhedrin promoter) were carried out as described [10,11]. The partial physical map of the transfer

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vector, pAc*βhCG-luc*, used for generating the recombinant vAc*βhCG-luc*, by in vivo recombination, is illustrated in Fig. 1. Recombinant virus stocks were amplified, purified and titrated [1].

2.2. *Detection and characterization of luc and βhCG*

Rearing and infection of *Spodoptera* larvae have been previously described [12]. Larvae were harvested 4 or 8 days post-infection (p.i.) and assayed for luc and βhCG. Total cell extracts of virus-infected caterpillars were prepared [12] and assayed for luc using X-ray film fogging assay [12,13]. Alternatively, larvae were bled by cutting off the prolegs, and hemolymph collected. Both hemolymph and the body tissue were assayed separately for luc and βhCG by SDS-PAGE and radioimmunoassay (RIA). For RIA [14,15], a monoclonal antibody specific to βhCG (NII Reagent Bank, New Delhi) was used. The biological activity of βhCG was determined by employing a mouse Leydig cell bioassay [16] with appropriate modifications [14,15].

2.3. *Western blot analysis*

Western blot of larval protein extracts [15] separated by SDS-PAGE was probed with an appropriate dilution of a polyclonal antiserum against luc, or with a monoclonal antibody against βhCG. Membranes were incubated in sheep anti-mouse IgG-HRP (horse radish peroxidase, Amersham, UK) or in goat anti-rabbit IgG-HRP (NII Reagent Bank, New Delhi) as the case may be. The enzymatic activity was revealed by colour development with freshly prepared 3,3′-diaminobenzidine solution.

3. RESULTS AND DISCUSSION

3.1. *Characterization of recombinant proteins*

Insect caterpillars have been demonstrated as excellent hosts for efficient synthesis of a foreign protein upon infection by a permissive baculovirus [8,12], and for the increased transcriptional activity of the polyhedrin promoter [17]. This study was aimed at exploring the stability of two recombinant proteins synthesized simultaneously in insect larvae infected with a double recombinant virus, vAc*βhCG-luc*. This virus was constructed by in vivo recombination [5] between the wild-type virus and a dual expression transfer vector, pAc*βhCG-luc*. In pAc*βhCG-luc*, constructed earlier [10], the cDNAs encoding βhCG and luc were cloned (Fig. 1) under the transcriptional control of independent copies of the AcNPV polyhedrin gene promoter placed in opposite orientations [7]. Recombinant baculovirus carrying a replacement of the native polyhedrin gene with the *βhCG-luc* cassette, was isolated and plaque purified [10]. The presence of both *βhCG* as well as *luc* genes in vAc*βhCG-luc* was confirmed by Southern hybridization (data not shown).

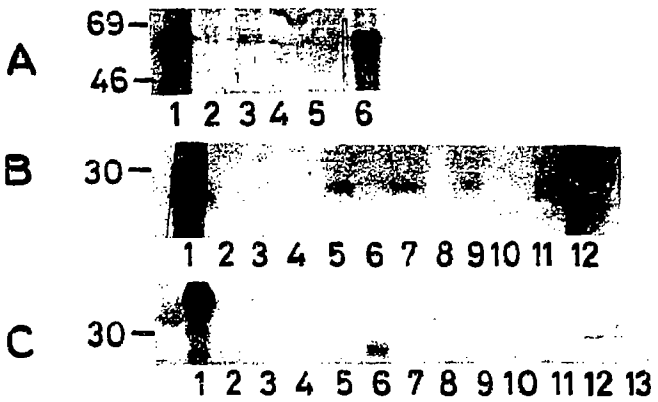

Fig. 2. Western blot of larval samples extracted 4 days p.i. (A) A luc-specific rabbit anti-luc polyclonal serum was used. Lanes 1,3, vAc*βhCG-luc*-infected larva (1, body tissue; 3, hemolymph); lane 2, mock-infected larva; lanes 4,5, AcNPV-infected larvae; lane 6, commercial preparation of firefly luc (2.5 μg). (B) The blot was probed with a monoclonal antibody against βhCG. Lanes 1,12, standard βhCG (>5 μg); lanes 2,3, AcNPV-infected larvae (2, body tissue; 3, hemolymph); lanes 4,6,8,10, mock-infected larvae (4,8, body tissue; 6,10, hemolymph); lanes 5,7,9,11, vAc*βhCG-luc*-infected larvae (5,11, hemolymph; 7,9, body tissue). (C) Comparative blot of infected cell and larval extracts. Sf9 cells were seeded in monolayer culture and infected with vAc*βhCG-luc* (at >0.5 MOI) as described [1]. Lane 1, standard βhCG; lanes 2,3, mock-infected Sf9 cells (2, cell pellet; 3, supernatant); lanes 4,7, AcNPV-infected Sf9 cells (4, cell pellet; 7, supernatant); lanes 5,6, vAc*βhCG-luc*-infected Sf9 cells (5, cell pellet; 6, supernatant); lanes 8,9, mock-infected larvae (8, body tissue; 9, hemolymph); lanes 10,11, AcNPV-infected larvae (10, body tissue; 11, hemolymph); lanes 12,13, vAc*βhCG-luc*-infected larvae (12, body tissue; 13, hemolymph).

Analysis of hemolymph and body tissue of *Spodoptera* larvae, infected with recombinant vAc*βhCG-luc*, revealed the synthesis of both the gene products, luc as well as βhCG. luc was identified on the basis of its cross-reactivity with a luc-specific rabbit anti-luc polyclonal serum on a Western blot (Fig. 2A). No immunoreactive material was present in mock-infected- or wild-type virus-infected larvae (Fig. 2A, lanes 2,4,5). Recombinant luc co-migrated with the standard firefly luc protein (Boehringer-Mannheim, Germany) at an apparent molecular weight of ~62 kDa. Densitometric scanning of SDS-PAGE revealed that ~15% of the total Coomassie blue-stainable protein of the infected larvae was represented by luc protein, while the βhCG level was <5% (data not shown).

Fig. 1. Partial physical map of the transfer vector pAc*βhCG-luc* showing the two polyhedrin promoter cassettes and the direction of transcription of the genes encoding βhCG and luc. Relevant restriction sites, used in checking the orientation of the insert with respect to the promoter, are indicated. Numbers against restriction enzyme sites reflect their relative position in the transfer vector with respect to nucleotide A within the *Eco*RI recognition sequence at the beginning of the AcNPV *Eco*RI 'I' fragment.

Recombinant βhCG, synthesized in infected larvae, was also identified by its cross-reactivity with a monospecific mouse anti-βhCG monoclonal antibody on a Western blot. Mock-infected- or wild-type-infected larval extracts did not contain any immunoreactive material (Fig. 2B, lanes 2–4,6,8,10). The recombinant βhCG was similar to native βhCG although it was hypoglycosylated, as evident from increased electrophoretic mobility. hCG is a heterodimer of α and β subunits containing ~33% carbohydrate. On SDS-PAGE, under reducing conditions, the native βhCG yields a molecular weight of 33 kDa [6]. The recombinant βhCG, with an apparent molecular weight of ~ 30 kDa, reacted with a mono-specific antibody (Fig. 2B, lanes 5,7,9,11) proving that the recombinant βhCG was similar to native βhCG but not identical. This indicated that insect caterpillars may not be capable of carrying out proper glycosylation of foreign proteins of mammalian origin to the extent present in the native protein. Insect cells have previously been shown to carry out glycosylation with a much lower efficiency [6]. However, a direct comparison of recombinant βhCG synthesized in insect cells and caterpillars revealed that the glycosylation was apparently more efficient in caterpillars than in cultured insect cells, as was evident from the reduced mobility of the βhCG band of caterpillar origin compared to that derived from insect cell culture (Fig. 2C, lanes 5,6,12,13). Besides a major βhCG band of ~30 kDa, the larval extract also yielded another slightly shorter (~27 kDa) band immunoreactive to anti-βhCG monoclonal antibody (Fig. 2B) which may possibly represent another population of the hypoglycosylated βhCG. The fact that both the ~30 kDa and ~27 kDa βhCG were immunoreactive implies that glycosylation had no significant effect on immunological properties of βhCG as previously reported [6].

The β subunit of hCG has six disulphide linkages involved in proper folding of the molecule. The individual subunits of hCG are not bioactive on their own and require an association with the corresponding subunit to form a dimeric hCG composed of α and β subunits which alone possesses biological activity [19]. The hemolymph and body tissue homogenate of infected larvae were, therefore, incubated with standard αhCG, and the extent of αβ dimer formation was estimated by a mouse Leydig cell bioassay [14–16]. Both hemolymph and body tissue from mock-infected- or wild-type virus-infected larvae did not exhibit biological activity either alone or in combination with externally added purified αhCG. Although βhCG was synthesized in vAc*βhCG-luc*-infected larvae, it did not exhibit biological activity, unless annealed in vitro to purified αhCG (Fig. 3). This assay clearly demonstrated the presence of biologically active βhCG in the hemolymph of infected larvae which could associate with standard αhCG to generate hormonal activity. However, there was a much reduced biological activity in the body tissue of larvae as compared to the hemolymph (Fig. 3). A direct comparison of the βhCG synthesized in insect cells and larvae revealed that ~45% of the recombinant protein from larvae was bioactive as compared to ~15–20% of bioactivity found in insect cell-synthesized βhCG [10,14].

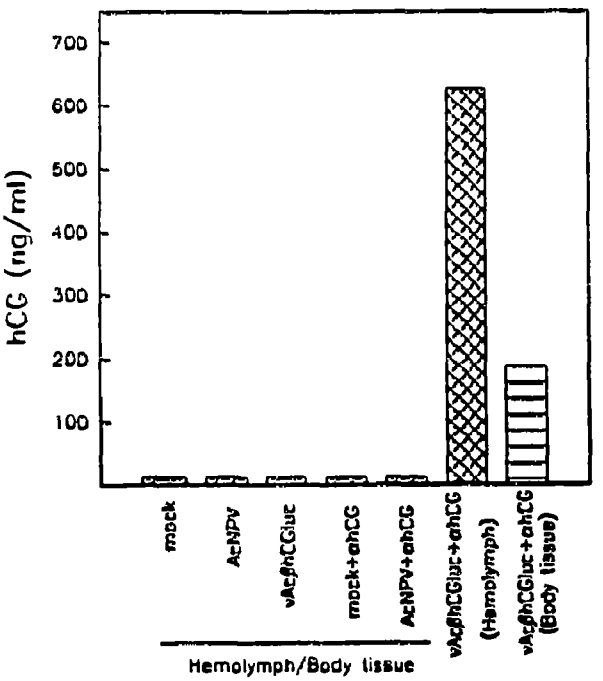

Fig. 3. Biological activity of βhCG synthesized in insect larvae 4 days p.i. Hemolymph and body tissue were incubated with a molar excess of purified standard αhCG for 16 h at 37°C and the αhCG/βhCG dimer was quantitated using a Leydig cell bioassay [14–16]. These results are the average of at least five experiments and variations between experiments were less than 10 ng.

3.2. *Secretion of recombinant proteins in infected larvae*

Insect cells can perform many of the post-translational modifications, such as glycosylation, secretion, proper protein folding [5,20], etc. In BEVS foreign proteins which do not enter the secretory pathway are synthesized to high levels (polyhedrin also does not enter the secretory pathway), often close to the level of polyhedrin, but secretory and membrane-bound glycoproteins are synthesized to levels which are at least an order of magnitude less [21]. Densitometric scanning of X-ray film, fogged due to the light emitted by enzymatically active luc, revealed that while most (~95%) activity was located in body tissue, a small fraction (~5%) was present in the hemolymph (Fig. 4). RIA confirmed the presence of recombinant βhCG in the infected larvae and indicated that the synthesis of βhCG increased with the time of infection, starting from 1,790 ng βhCG/larva (hemolymph and body tissue) at 4 days p.i., and increasing to 2,640 ng/larva (1,200 and 1,440 ng, respectively, in hemolymph and body tissue) at 8 days p.i. Our results also support the apparent bias against secretory protein

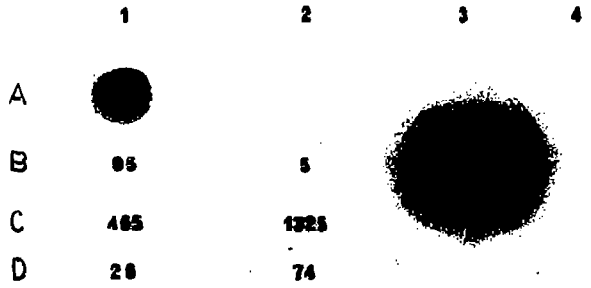

Fig. 4. Comparison of luciferase and βhCG synthesized in the vAcβhCG-*luc*-infected larva 4 days p.i. luc was detected by fogging of X-ray film as described [12,13] while βhCG was estimated by RIA [14–16]. (A) Lanes 1,2, vAcβhCG-*luc*-infected larva (1, body tissue; 2, hemolymph); lanes 3,4, mock-infected larva (3, body tissue; 4, hemolymph); lane 3 of panel B shows standard luc (1 μg). (C) βhCG (ng) synthesized in larva (lane 1, body tissue; 2, hemolymph). B and D (lanes 1 and 2) give the relative percentage of recombinant proteins in corresponding lanes of A and C, respectively.

in BEVS. luc, a non-secretory protein, was synthesized to much higher levels than the secretory βhCG protein in insect larvae though both were under identical transcriptional controls.

While most of the luc was retained in body tissue, SDS-PAGE and Western blots revealed the presence of luc in hemolymph as well. Hemolymph contains hemocytes, a cell type which is indeed infected with baculovirus. Previous studies indicate that the fat body of larvae synthesizes and releases protein into the hemolymph and also takes up protein from the hemolymph [22]. Other tissues, such as midgut, epidermis, pericardial cells and hemocytes also contribute to hemolymph proteins [23]. Presence of luc in hemolymph, thus, could be explained on these lines. Nevertheless, enzymatically active luc appears to be synthesized throughout the entire larva, which is evident from the total fluorescence of the recombinant virus-infected caterpillar upon injection of luciferin (data not shown). βhCG, on the other hand, is an extensively glycosylated secretory protein and was efficiently secreted into larval hemolymph, as evident from RIA and Western blot analyses. The secreted βhCG was biologically active but the body tissue-associated βhCG had reduced bioactivity. It is possible that the folding of the secreted βhCG into the native conformation was complete and accurate after which it was released into the hemolymph while that in the body tissue was partially folded or represented a mixture of different species, mainly non-bioactive forms.

Interestingly, a large majority of the recombinant virus-infected larvae did not molt into pupa, and remained in larval stage till their death due to the arrest of metamorphosis caused by the viral *egt* gene [24]. The prolonged life span of infected larvae by 4–5 days is of special relevance for harvesting of recombinant proteins from larvae. Most, if not all, foreign proteins synthesized during the larval stage naturally have sufficient time to undergo proper post-translational modifications. We previously reported [15] a secretory load on the insect cells to process the abundantly synthesized hCG protein within the short time available to the cells due to the lytic nature of the virus. The natural extension of the life span of the larvae upon infection, therefore, circumvents this problem. The reduced mobility and increased bioactivity of the larval-synthesized hCG, as compared to the insect cell culture-derived protein, are possibly the consequence of more time available to process this complex protein.

Results presented above demonstrate that a recombinant baculovirus derived from a transfer vector containing duplicate copies of the polyhedrin promoter is genetically stable and capable of producing, in caterpillars, two foreign proteins simultaneously. The similarity of recombinant luc and βhCG with native proteins as described above points to the fact that the recombinant proteins synthesized in the infected larvae are stable and folded into a conformation perhaps characteristic of the native proteins. Biological and immunological properties clearly indicate that they are of potential utility in biochemical, immunodiagnostic and clinical applications. The fact that the cost of rearing caterpillars is nominal makes the synthesis of such biomolecules economically viable.

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