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Expression of the Glycoprotein Gene from a Fish Rhabdovirus by Using Baculovirus Vectors†

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A cDNA fragment containing the gene encoding the glycoprotein of infectious hematopoietic necrosis virus was inserted into *Autographa californica* baculovirus vectors under the control of the polyhedrin promoter. A 66-kilodalton protein, identical in size to the glycosylated glycoprotein of infectious hematopoietic necrosis virus, was expressed at high levels in *Spodoptera frugiperda* cells infected with the recombinant viruses. The expressed protein reacted with antiserum to the glycoprotein on Western blots (immunoblots).

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus which infects young salmon and trout (9). Over the last 30 years, IHNV has caused extensive mortality in hatchery fish populations in the Pacific Northwest of the United States. Currently, control methods for the disease are limited to avoidance procedures which consist of the destruction of the infected fish and quarantine of all other fish in the hatchery.

The development of a vaccine against IHNV would be of great practical and economical value. The safety of live attenuated vaccines has been questioned in the case of IHNV because virus transmission occurs via the water (8). Since the virion surface glycoprotein of IHNV is immunoprotective (1), a subunit vaccine consisting of the glycoprotein was considered as an alternative.

In a previous report, we described the cloning and sequencing of a cDNA of the IHNV glycoprotein. The cDNA of the IHNV glycoprotein gene is 1,609 base pairs long and encodes a protein of 508 amino acids, which translates into a molecular weight of 56,795 (3).

Recombinant baculoviruses are capable of expressing proteins at high levels (6). In addition, because expression occurs in insect cells, the proteins appear to be processed in a eucaryotic manner. To determine if the baculovirus system is capable of expressing the IHNV glycoprotein, we have constructed recombinant baculoviruses carrying the IHNV glycoprotein gene.

The ability of baculovirus expression systems to produce IHNV glycoprotein was assessed with four recombinant baculoviruses. The IHNV glycoprotein gene was ligated into the transfer vectors pAc611 and pAc373 that were provided by M. D. Summers, Department of Entomology, Texas A & M University, College Station, Tex. The following constructions were used (Fig. 1): pAc611-G1, pAc611-G2, pAc373-G3, and pAc373-G4. In constructing pAc611-G1, the glycoprotein gene was excised from the pT7-2 plasmid with *Pst*I and ligated into the *Pst*I site of the vector pAc611. This construct contained the entire glycoprotein cDNA, including 12 G residues from the cDNA cloning protocol and an untranslated leader sequence of 48 nucleotides at the 5′ end. The clones pAc611-G2, pAc373-G3, and pAc373-G4 were constructed to eliminate these untranslated sequences, because it was thought that they might inhibit efficient expression of the glycoprotein. In constructing pAc611-G2, the glycoprotein gene was removed from its parent plasmid with *Bst*XI and *Hin*dIII and cloned into the *Sma*I site of pAc611. The *Bst*XI site was located at the first ATG of the glycoprotein coding sequence. In constructing pAc373-G3, the glycoprotein gene was first subcloned into the plasmid vector pUC19 and, in a second cloning step, it was put into the vector pAc373. In constructing pAc373-G4, the glycoprotein gene was cut with *Bst*XI and *Hin*dIII and blunt ends were created to clone it into the unique *Bam*HI site of pAc373.

In all four plasmid constructions, the 3′ end was cut from inside the parent plasmid pT7-2 with either *Pst*I or *Hin*dIII, leaving a noncoding region of 34 nucleotides after the TAA stop codon at position 1573. This stop codon should be utilized in the baculovirus system. However, changes in the 5′ end of the glycoprotein gene might affect the expression process and expression levels (6). The 5′ end of the glycoprotein gene was different in each of the four plasmid constructions. The recombinant plasmid, pAc611-G1, has 12 G residues at the 5′ end followed by a noncoding region of 48 nucleotides, and the two ATG sites at positions 49 and 58 are the same as in the original cDNA clone (ACAATGGACAC CATGA) (3).

In the three other plasmid constructions, the 5′ end occurred at the *Bst*XI site of the glycoprotein gene so that the noncoding nucleotide residues were removed and the glycoprotein gene sequence started at the first ATG. When ligated into the *Sma*I site of pAc611 or pUC19, the 5′ end sequence would read CCCATGGACACCATGA. This is the case for pAc611-G2 and pAc373-G3. The fourth plasmid construction, pAc373-G4, has the glycoprotein gene cloned into the blunt end of the *Bam*HI site and its 5′ start sequence was GGATCATGGACACCATGA. Both ATG codons at the beginning of the glycoprotein gene reside at the same reading frame and the initial ATG is the presumptive start codon. When the first ATG start sites for each plasmid construction were analyzed for the consensus translational initiation sequence, ACCATGG (4), all had the requisite G in the +4 position. pAc611-G1 and pAc373-G4 have a purine (A) in the −3 position which is favored over the C in the −3 position in the two other plasmid constructions. This analysis indicated that expression of the IHNV glycoprotein in the baculovirus system with these plasmids was theoretically possible.

The IHNV glycoprotein gene in the recombinant plasmid was then transferred into the wild-type *Autographica cali-*

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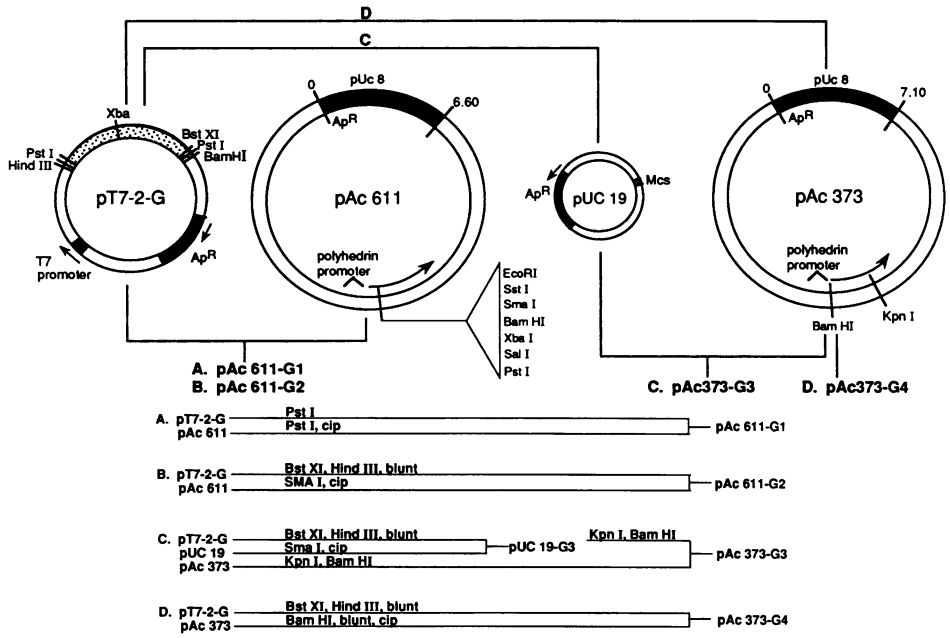

FIG. 1. Construction of recombinant baculovirus vectors containing the glycoprotein gene of infectious hematopoietic necrosis virus. pAc611-G1 (A) contains the glycoprotein gene in the *Pst*I site of pAc611. pAc611-G2 (B) contains the glycoprotein gene in the *Sma*I site of pAc611. pAc373-G3 (C) contains the glycoprotein gene between the *Bam*HI and *Kpn*I sites of pAc373. pAc373-G4 (D) contains the glycoprotein gene in the *Bam*HI site of pAc373. The restriction maps of pAc611 and pAc373 were modified from versions of those described by Summers and Smith (10). ▨, Glycoprotein gene; ■, ampicillin resistance gene and T_7 promoter.

fornica nuclear polyhedrosis virus (Ac*M*NPV) genome by homologous recombination within insect cells. To accomplish this, *S. frugiperda* Sf9 cells were cotransfected with 2 μg of Ac*M*NPV DNA and 3 μg each of the recombinant baculovirus vectors (10). Plaques produced from each transfection were screened under the microscope for polyhedrin-negative phenotypes. Plaque hybridizations with a glycoprotein gene-specific probe labeled with ^{32}P by nick translation were performed to confirm the results. After a second cycle of plaque purification and plaque hybridization, recombinant viruses containing the glycoprotein gene were isolated from the infected insect cells and stocks of the recombinant viruses were obtained through infection of insect cells.

Monolayers of *S. frugiperda* cells were infected at high multiplicity with Ac*M*NPV or the recombinant baculoviruses. At 3 and 6 days postinfection, the infected cells were lysed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5) (Fig. 2). All four recombinants synthesized a major protein species that migrated with an estimated size of 66,000 daltons, similar to that of the IHNV glycoprotein (7). A densitometer scan of the gel shown in Fig. 2 indicated that the quantity of glycoprotein produced by infected cells was 20 to 30% of the total protein (data not shown).

The virus-produced protein was analyzed by immunoblotting with rabbit anti-IHNV glycoprotein serum (Fig. 3). Lanes containing expressed glycoprotein all reacted positively with the antiserum, whereas lanes containing mock-infected or Ac*M*NPV-infected samples did not show a reaction with the antiserum. The size of the protein as determined by gel electrophoresis corresponded to the size of the glycosylated form of the glycoprotein (molecular weight, 66,000), as opposed to the molecular weight of the nonglycosylated form, whose predicted molecular weight was 56,795. There are five potential *N* glycosylation sites in the glycoprotein DNA sequence (3). The vector difference as well as the differences in the 5′ ends of the constructs appeared to have little or no effect on the level of expression of the glycoprotein in this system.

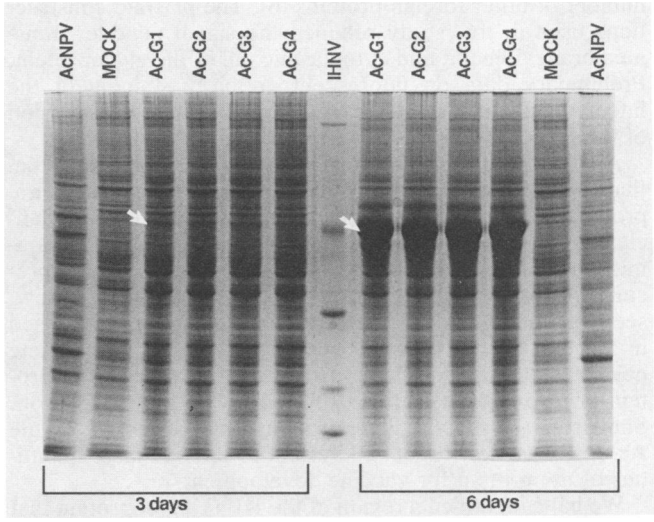

FIG. 2. Analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *S. frugiperda* cells were infected with the following recombinant baculoviruses: Ac611-G1, Ac611-G2, Ac373-G3 and Ac373-G4. Cell lysates were analyzed by electrophoresis on a 9% polyacrylamide gel (5) and stained with Coomassie brilliant blue. Samples of cells infected with wild-type Ac*M*NPV and mock-infected cells were included for comparison. Lane 7 (designated IHNV) contains purified IHNV proteins whose molecular weights are in the following order of size: L, 150,000; G, 66,000; N, 40,000; M1, 25,000; and M2, 21,000. The arrows indicate the positions of the expressed IHNV glycoprotein.

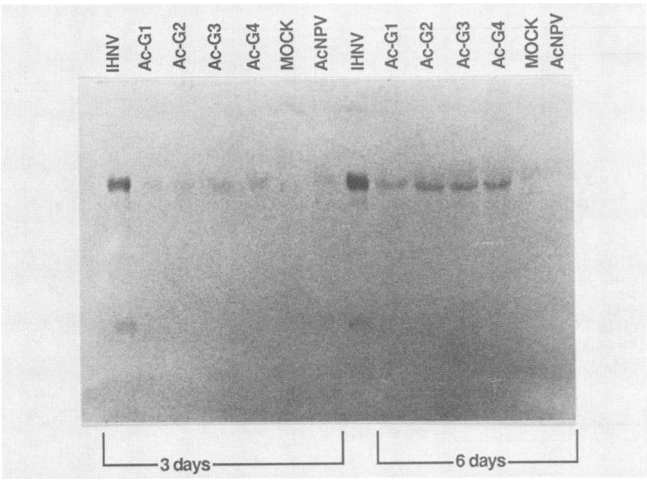

FIG. 3. Analysis of proteins by Western immunoblot. *S. frugiperda* cells were infected with recombinant or wild-type Ac*M*NPV or were mock infected as described in the legend to Fig. 2. In this gel, the samples for the 6-day cell lysates contained 1/10 of that represented in Fig. 2. Purified IHNV proteins were included as a positive control. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane. The blot was incubated with a 1:250 dilution of rabbit anti-IHNV glycoprotein serum. The bound antibody was detected by using goat anti-rabbit immunoglobulin G horseradish peroxidase complex (Sigma Chemical Co., St. Louis, Mo.) with 4-chloro-1-naphthol as substrate.

Immunoprecipitation experiments showed that the glycoprotein was not present in detectable amounts in the tissue culture supernatant (data not shown). Only cell lysates contained the expressed protein. This finding suggested that the glycosylated glycoprotein was not secreted by the insect cells. It was more likely that the expressed protein remained on the infected cell surface, as has been reported for a number of other foreign proteins (6). The plasmid constructions used in this study retained the signal peptide, transmembrane domain, and cytoplasmic tail of the glycoprotein. Preliminary data on fluorescent antibody staining of the infected cells with anti-IHNV sera show surface expression of the glycoprotein.

Among the proteins expressed in the baculovirus system that undergo subsequent *N*-glycosylation were many viral proteins such as the glycoprotein of human immunodeficiency virus, the hemagglutinin of influenza virus, the neuraminidase of parainfluenza virus, and the glycoprotein precursor of lymphocytic choriomeningitis virus (for a review, see reference 6). All of these glycoproteins were expressed in the baculovirus system because it provided appropriate posttranslational modifications and expressed levels of protein of up to 500 μg/ml per 10^6 cells at 72 h postinfection. Similarly, the IHNV glycoprotein was expressed in the Ac*M*NPV vector-insect cell system to provide large quantities of the protein for vaccine development.

We have identified a region of the IHNV glycoprotein that induces protective immunity in young salmon and trout (2). Although this vaccine (a crude extract of *Escherichia coli* harboring a plasmid expressing a trpE-glycoprotein segment fusion protein) was effective with many different strains of the virus, only a small segment of 350 bases from the glycoprotein gene was expressed. The 16 cysteine residues present in the glycoprotein made expression of the entire protein in native form in *E. coli* unlikely. Certain virus strains might not contain epitopes cross-reactive with this region of the gene. Thus, the synthesis of the entire IHNV glycoprotein in glycosylated form in insect cells makes possible the development of a more complete IHNV vaccine. The efficacy of the recombinant baculovirus-produced glycoprotein as a vaccine has yet to be tested in vivo.

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