

## Production of Human Beta Interferon in Insect Cells Infected with a Baculovirus Expression Vector

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*Autographa californica* nuclear polyhedrosis virus (AcNPV) was used as an expression vector for human beta interferon. By using specially constructed plasmids, the protein-coding sequences for interferon were linked to the AcNPV promoter for the gene encoding for polyhedrin, the major occlusion protein. The interferon gene was inserted at various locations relative to the AcNPV polyhedrin transcriptional and translational signals, and the interferon-polyhedrin hybrid genes were transferred to infectious AcNPV expression vectors. Biologically active interferon was produced, and greater than 95% was secreted from infected insect cells. A maximum of ca.  $5 \times 10^6$  U of interferon activity was produced by  $10^6$  infected cells. These results demonstrate that AcNPV should be suitable for use as a eucaryotic expression vector for the production of products from cloned genes.

*Autographa californica* nuclear polyhedrosis virus (AcNPV) has a genome of ca. 130 kilobases of double-stranded, circular DNA and is the most extensively studied baculovirus (for recent reviews see references 13 and 20). Its host range is limited to lepidoptera species and cultured lepidoptera cells, and it is currently being considered for agricultural use as a viral insecticide. AcNPV has a biphasic replication cycle and produces a different form of infectious virus during each phase. Between 10 and 24 h postinfection (p.i.), extracellular virus is produced by the budding of nucleocapsids through the cytoplasmic membrane. By 15 to 18 h p.i., nucleocapsids are enveloped within the nucleus and viral occlusions begin to form. Each occlusion contains many virus particles embedded in a paracrystalline protein matrix, which is formed from a single major protein called polyhedrin. In infected *Spodoptera frugiperda* (fall armyworm, Lepidoptera, Noctuidae) cells, AcNPV polyhedrin accumulates to high levels and constitutes 25% or more of the total protein mass in the cell (34); it is probably synthesized in greater abundance than any other protein in a virus-infected eucaryotic cell.

Polyhedrin is encoded by the virus (40), and the gene has been mapped (34) and sequenced (B. J. L. Hooft van Iddekinge, G. E. Smith, and M. D. Summers, *Virology*, in press). From an analysis of viral deletion mutants, we demonstrated that the polyhedrin gene is not required for the production of infectious extracellular virus (30). Inactivation of the polyhedrin gene by

deletion (30) or, as will be demonstrated in this report, by insertion results in mutants that do not produce occlusions in infected cells. These occlusion-negative viruses form plaques that are different from plaques produced by wild-type viruses, and this distinctive plaque morphology is used as a means to screen for recombinant viruses (30).

Several properties of AcNPV that may make this virus ideally suited as an expression vector for cloned eucaryotic genes have been described (20). These include the potential of the rod-shaped virus to encapsidate viral genomes with large pieces of additional, foreign DNA and the inherent safety of a recombinant viral vector that is not pathogenic to vertebrates. In addition, the polyhedrin gene provides: (i) a nonessential region of the AcNPV genome in which to insert foreign DNA, (ii) a very strong promoter which directs transcription late in infection after extracellular virus is produced and after host genes and most viral genes are turned off, and (iii) a genetic marker to select for recombinant viruses. To examine the utility of AcNPV as an expression vector, we fused the gene for human beta interferon (IFN- $\beta$ ) to the AcNPV polyhedrin promoter and measured its production in infected cells.

IFN- $\beta$  is known to have antiviral, antiproliferative, and antitumor properties and should prove important in a variety of medical applications. Although cultured human cells normally do not produce interferon, treatment with inducers such as viruses or double-stranded RNA

results in a transient and relatively low-level biosynthesis of IFN- $\beta$  (35). The IFN- $\beta$  gene has been expressed in *Escherichia coli* cells by fusing interferon protein-coding sequences to bacterial promoter and ribosomal binding sites in plasmid (8, 37) or lambda phage (22) vectors. Nonhuman mammalian cells have also been employed to synthesize IFN- $\beta$  from the cloned gene. For example, the IFN- $\beta$  gene has been cloned with its protein-coding sequences under the transcriptional control of a simian virus 40 promoter (7), a herpes simplex virus thymidine kinase promoter (25), or its own genetic signals (2, 11, 21, 24, 43). The highest reported levels of beta interferon activity produced in bacterial and animal host-vector systems are  $1 \times 10^4$  U/ml in *E. coli* cells (8) and  $5 \times 10^4$  U/ml in transformed mouse cells (11). By using AcNPV as an expression vector, biologically active IFN- $\beta$  was efficiently secreted in the media of infected cells and reached a titer of  $5 \times 10^6$  U/ml. In addition, the IFN- $\beta$  protein was glycosylated, and our data indicate that the signal peptide at the amino-terminal end of the primary translation product was removed.

#### MATERIALS AND METHODS

**Cells and virus.** AcNPV stocks were grown and titers were determined in *S. frugiperda* cells (IPLB-SF21-AE), using Hink medium (12) plus 10% fetal bovine serum (FBS) as described previously (31).

Human amniotic (WISH) cells were maintained in Earle minimum essential medium supplemented with penicillin (100 U/ml), mycostatin (50 U/ml), and 15% FBS.

**Interferon.** Interferon activity was determined by the virus plaque-reduction assay (18) in WISH cells challenged with vesicular stomatitis virus. Serial dilutions (0.5 log) of culture media from recombinant AcNPV-infected cells were made over  $2.5 \times 10^4$  WISH cells in 96-well microtiter plates. After 12 h at 37°C, each well was infected with ca. 60 PFU of vesicular stomatitis virus, and the cells were overlaid with 0.5% methyl cellulose in Earle minimum essential medium. At 24 h p.i., the cell monolayers were stained with crystal violet and the plaques were counted. An international reference standard of human interferon was included in all assays, and the activity was expressed in National Institutes of Health reference units (IU) per milliliter.

**Transfection and selection for recombinant viruses.** The procedure used to transfect *S. frugiperda* cells and the marker-transfer method used to select for recombinant AcNPV expression vectors were as described previously (30).

**Protein blot radioimmunoassay.** Total cellular proteins were electrophoretically separated and then reacted with antibody as described previously (32). At 30 h p.i., cells were washed with  $1 \times$  phosphate-buffered saline and ca. 100- $\mu$ g samples of cell protein were electrophoresed on a sodium dodecyl sulfate-12% polyacrylamide gel (12) and then transferred by diffusion to two nitrocellulose filters. The filters were incubated with either a 1:1,000 dilution of rabbit

antiserum prepared against AcNPV polyhedrin (32) or a 10- $\mu$ g/ml concentration of monoclonal antibody to recombinant IFN- $\beta$  (Hoffmann-La Roche Inc., Nutley, N.J.). Specifically bound antibody was detected by using radioiodinated *Staphylococcus aureus* protein A.

**Radiolabeling of infected cells and analysis of polypeptides.** At various times after infection, cells were labeled for 30 min in leucine-free Grace medium (9) supplemented with 2% dialyzed FBS and L-[ $^3$ H]leucine at 100  $\mu$ Ci/ml or for 60 min in Grace medium with 10% the normal glucose supplemented with 2% dialyzed FBS and D-[ $^3$ H]mannose at 200  $\mu$ Ci/ml. To label cells in the presence of tunicamycin, infected cells were pretreated for 20 to 30 h p.i. with 5  $\mu$ g of tunicamycin per ml and then labeled as described above with L-[ $^3$ H]leucine in the presence of 5  $\mu$ g of tunicamycin per ml. The labeled cells were washed with Grace medium, disrupted and electrophoresed on sodium dodecyl sulfate-12% polyacrylamide gels, and then processed for fluorography as described previously (33). Molecular weights were calculated by comparing electrophoretic mobility with that of protein markers (Pharmacia Fine Chemicals, Piscataway, N.J.).

**Construction of AcNPV transfer vectors.** AcNPV *EcoRI*-I, which contains the polyhedrin gene (1, 26, 33), was cloned into the *EcoRI* site of pUC8 (41). The resulting plasmid (p18) has three *Bam*HI recognition sites: one in the polyhedrin gene, one in *EcoRI*-I downstream from the polyhedrin gene, and one in the cloning region of pUC8. The *Bam*HI site in pUC8 was removed by digesting p18 successively with *Pst*I, *Sma*I, and S1 nuclease. *E. coli* JM83 cells were transformed with the digested DNA, and a plasmid was isolated that was missing *Pst*I, *Bam*HI, *Sma*I, and *EcoRI* restriction sites in the cloning region of pUC8. This plasmid was partially digested with *Bam*HI, and full-length linear DNA was isolated from an agarose gel, treated with S1 nuclease (500 U/ml) at 37°C for 15 min, circularized with T4 DNA ligase, and used to transform JM83 cells. A plasmid (pAc101) was identified with a single *Bam*HI site located ca. 220 bases from the polyhedrin transcriptional start site (Fig. 1; 34).

The following procedure was used to modify pAc101 such that some or all of the DNA sequences between the transcriptional start site and the *Bam*HI recognition sequence in the polyhedrin gene were deleted and replaced by a synthetic *Bam*HI linker sequence, 5'-dCGGATCCG-3' (P-L Biochemicals, Inc., Milwaukee, Wis.). The *EcoRI*-to-*Bam*HI fragment in AcNPV *EcoRI*-I (located 0 to 4,210 bases in *EcoRI*-I; see reference 34) was cloned into pUC8. Forty micrograms of this plasmid (pB') was digested with *Bam*HI and then incubated for 40 min at 30°C with 0.5 U of *Bal* 31 exonuclease. At 4-min intervals, 10- $\mu$ l samples were removed, and the reaction was stopped by adding 10  $\mu$ l of 0.025 M EDTA. The samples were pooled, phenol extracted, and precipitated with 2 volumes of ethanol. The ends of the DNA were repaired by incubating the DNA in 100  $\mu$ l for 30 min at 23°C with 4 U of *E. coli* DNA polymerase (Klenow fragment). Phosphorylated *Bam*HI linkers were added to the ends with T4 DNA ligase, the fragments were digested with *Bam*HI and *EcoRI*, and truncated pB' fragments with ca. 100- to 400-base-pair deletions were isolated from

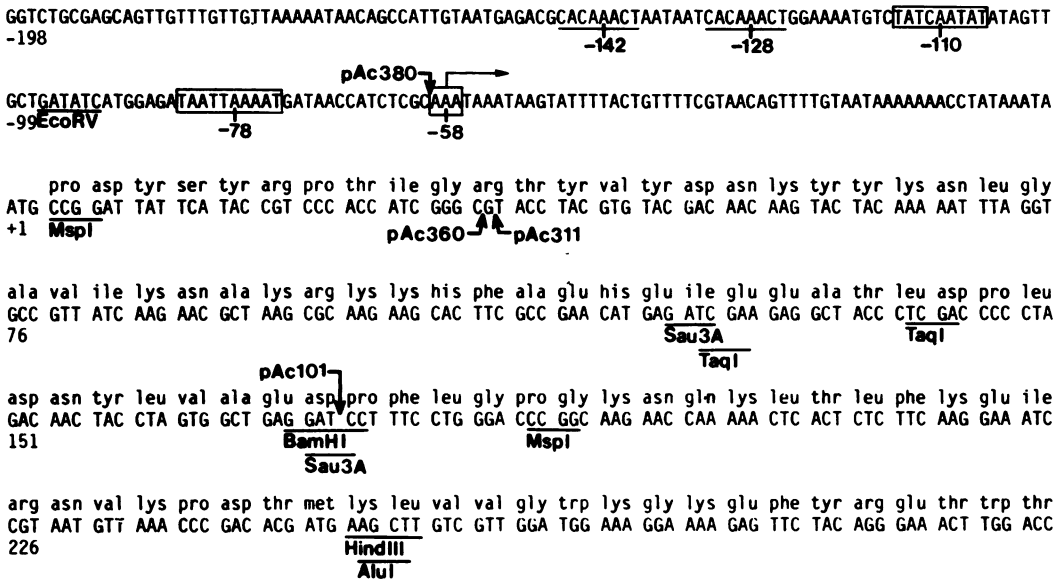


FIG. 1. Nucleotide and amino sequence of the 5' end of the polyhedrin gene. The DNA sequence of a segment of the polyhedrin gene and 5' flanking region is presented as the sense (+) strand and was determined using the dideoxy termination procedure (29). The sequence around the 5' end of the polyhedrin gene was determined from the following overlapping templates: the minus strand beginning at the *HindIII* or *BamHI* sites in pAc101, the minus strand beginning at the synthetic *BamHI* sites in pAc311, pAc360, and pAc380, and the plus strand beginning at the *BamHI* or *HindIII* sites in pAc101 (Fig. 2). The location of the natural *BamHI* site in the transfer vector pAc101 and positions of the *BamHI* linkers in pAc311, pAc360, and pAc380 are indicated by arrows. The box at -58 marks the bases that code for the 5' end of polyhedrin mRNA (34). The rectangles centered at -78 and -110 mark the nucleotides that resemble the canonical TATA and CAAT box sequences, respectively. The repeated CACAACT sequences upstream from the CAAT box are underlined. The translational start signal for polyhedrin is assigned position +1.

an agarose gel. The purified fragments were ligated to *EcoRI*- plus-*BamHI*-digested pUC8 and then used to transform JM83 cells. The sizes of the deletions in the resulting clones were estimated by restriction enzyme analysis and in some cases by DNA sequencing. The *XhoI*-to-*BamHI* fragment in pAc101 (located at 1,900 to 4,210 bases in *EcoRI*-I; 34) was removed and replaced with *XhoI*-to-*BamHI* linker fragments from several different pB' mutant plasmids, using standard cloning procedures, to produce the modified transfer vectors pAc311, pAc360, and pAc380. These plasmids have single *BamHI* cloning sites in the polyhedrin gene at the locations indicated in Fig. 1.

**DNA sequencing.** AcNPV restriction fragments were cloned into M13mp8 or M13mp9 (19), and the single-stranded recombinant phage DNAs were purified and used as templates in the chain-termination sequencing method (29). The sequence was determined from overlapping DNA templates, and all autoradiographs were read independently at least twice. The sequence across all restriction enzyme sites in the polyhedrin gene was also determined.

## RESULTS

**Nucleotide sequence of the polyhedrin promoter and the construction of transfer vectors.** The nucleotide sequence of the 5' end of the polyhedrin-coding region and 200 bases upstream from

the start of translation is shown in Fig. 1. The site that specifies the 5' end of polyhedrin mRNA has been mapped in a high-resolution S1 nuclease experiment to 220 bases in the 5' direction from the single *BamHI* site that maps within the gene (Fig. 1; 34). An ATG translational start signal (the A residue was assigned position +1) was located ca. 58 bases from the transcription start site, followed by an open reading frame for 244 amino acids. Proline is known to be the amino-terminal residue for AcNPV polyhedrin (J. Maruniak, Ph.D. thesis, University of Texas, Austin, 1980) and was the penultimate N-terminal amino acid predicted from the DNA sequence (Fig. 1). The predicted sequence for AcNPV polyhedrin (Fig. 1) was 80 to 90% homologous with several other NPV polyhedrins (28). The complete sequence of the AcNPV polyhedrin gene will be presented in another report (Hooft van Iddekinge et al., in press). Located about 78 and 110 bases upstream from the translational start site were sequences that resembled, respectively, the canonical "TATA" and "CAAT" boxes found in similar positions in many eucaryotic structural genes (see reference 5 and references therein). Centered at -128 and -142 were the direct

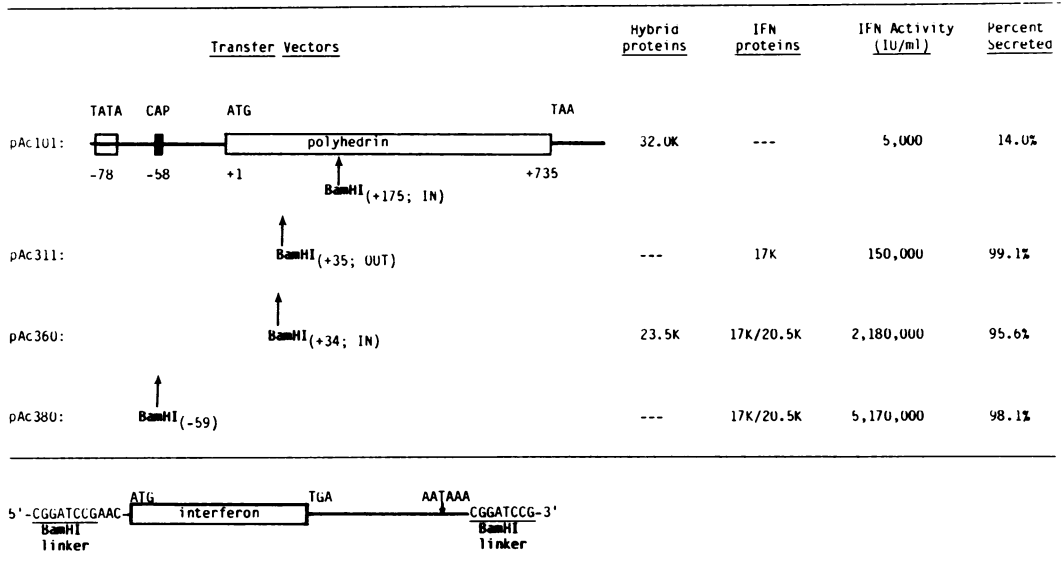


FIG. 2. Location of *Bam*HI cloning sites in the transfer vectors pAc101, pAc311, pAc360, and pAc380 illustrated relative to the coding sequences (open rectangles), the putative cap site, and the TATA box sequences of the polyhedrin gene. The interferon fragment cloned into these *Bam*HI sites is shown in the lower panel. When cloned into pAc101 and pAc360, the interferon coding sequences (open rectangle) are in the same reading frame as polyhedrin, and when cloned into pAc311 they are not. The polyhedrin-interferon hybrid proteins and 17K non-glycosylated and 20.5K glycosylated IFN- $\beta$  proteins produced by AcNPV expression vectors are indicated. Interferon was produced by AcNPV expression vectors, and the activity was measured as follows. *S. frugiperda* cells were infected at 20 PFU per cell with AcNPV or AcNPV expression vectors. For infection,  $4 \times 10^6$  *S. frugiperda* cells were seeded in 4 ml of medium in a 25-cm<sup>2</sup> culture flask and then infected for 1 h at 23°C with 1 ml of virus inoculum. Infected cells were washed, 4 ml of fresh medium plus 10% FBS were added, and the flasks were incubated at 27°C for 48 h. The media were removed and centrifuged for 10 min at  $3,000 \times g$ . The supernatant from each was centrifuged at  $100,000 \times g$  for 30 min at 4°C and then stored at -80°C until assayed. Infected cells were washed in phosphate-buffered saline, suspended at  $2 \times 10^6$  cells per ml, and then disrupted by mixing with an equal volume of 7 M guanidine hydrochloride. Media and disrupted cells were assayed for interferon by the plaque-reduction assay and compared with an international interferon reference standard (18).

repeated nucleotides CACAAACT. These sequences may be analogous to the tandemly repeated promoter element (-100 region) in, for example, the promoter region of rabbit beta globin (5). The AcNPV polyhedrin gene has a 58-base nontranslated leader sequence preceding the translational start codon, and, as suggested from S1 nuclease experiments of AcNPV polyhedrin mRNA (34) and R-loop mapping of another NPV polyhedrin mRNA (27), these were no intervening sequences.

The AcNPV genome has no known unique restriction sites into which genes can be conveniently introduced in a site-specific manner. To incorporate the gene for IFN- $\beta$  into the viral genome adjacent to the polyhedrin promoter, we first constructed chimeric plasmid vectors (transfer vectors). Each of these has both a cloning site near the polyhedrin promoter and flanking viral DNA linked to the *E. coli* plasmid pUC8. *Eco*RI fragment I was cloned into the *Eco*RI site in pUC8. There are three *Bam*HI sites on this plasmid, one of which is located at

position +175 in the polyhedrin gene (Fig. 1). The other two *Bam*HI sites were removed, resulting in the transfer vector pAc101. From the nucleotide sequence of polyhedrin and interferon, it can be determined that the IFN- $\beta$  protein-coding sequences, when inserted into the unique *Bam*HI cloning site in pAc101, would be in the same translational reading frame as polyhedrin.

To investigate the importance of various polyhedrin gene sequences in the expression of IFN- $\beta$ , the transfer vector pAc101 was modified to have *Bam*HI cloning sites at other positions near the 5' end of the polyhedrin gene. Deletions were produced between the natural *Bam*HI site at +175 and the polyhedrin promoter in pAc101, and a synthetic oligonucleotide containing a *Bam*HI recognition site was added at the points of deletion. The transfer vectors pAc311 and pAc360 have *Bam*HI sites at +35 and +34 bases, respectively, downstream from the start site of polyhedrin translation (Fig. 1). The vector pAc380 has a *Bam*HI cloning site at -59, which

is the putative cap site for polyhedrin mRNA (Fig. 1).

The nucleotide sequence for IFN- $\beta$  and the location of various transcription and translation signals are known (4, 10, 23). A 767-base-pair *HincII* fragment from a genomic clone of human IFN- $\beta$  (pBR13; 11) (containing the entire protein coding sequences for IFN- $\beta$ , three additional bases before the ATG translation start signal, and all of the nontranslated 3' sequences including the signal for polyadenylation [23]) was cloned into the *HincII* site of pUC8. This fragment was joined to synthetic octanucleotide *Bam*HI linkers and then cloned into the unique *Bam*HI sites in pAc101, pAc311, pAc360, and pAc380 (Fig. 2). Plasmids were examined, using various restriction enzymes to obtain the transfer vectors in which IFN- $\beta$  was oriented in the same 5' to 3' direction as the polyhedrin gene, hereafter referred to as pAc101-IFN- $\beta$ , pAc311-IFN- $\beta$ , pAc360-IFN- $\beta$ , and pAc380-IFN- $\beta$ . As an example, pAc380 and the location and orientation of the interferon fragment in this transfer vector are shown in Fig. 3.

**Construction of AcNPV expression vectors.** Next, we transferred these hybrid polyhedrin-interferon genes to the AcNPV genome. AcNPV DNA was mixed with each of the transfer vectors containing the hybrid genes, and *S. frugiperda* cells were transfected as described previously (30). Recombinant AcNPV with insertions of IFN- $\beta$  DNA was obtained from the resulting viral progeny by screening for plaques formed by viruses that did not produce occlusions ( $O^-$  mutants). Of the viral plaques resulting from the virus produced in cells cotransfected with viral and plasmid DNAs, an average of 0.5% were formed from putative recombinant  $O^-$  viruses. DNA restriction analysis demonstrated that each had the addition of IFN- $\beta$  DNA at the appropriate location in *Eco*RI fragment I and that no other changes in the viral DNAs were detected (data not shown). The recombinant viruses were, therefore, defective in the production of occlusions due to insertional inactivation of the polyhedrin gene. Referred to here as the viral expression vectors Ac101-IFN- $\beta$ , Ac311-IFN- $\beta$ , Ac360-IFN- $\beta$ , and Ac380-IFN- $\beta$  they resulted from recombination between AcNPV and the corresponding IFN- $\beta$  transfer vectors. The details of the relatively simple screening procedure used to select for AcNPV recombinants are described in a recent report (30).

**Biologically active interferon is produced and secreted in cells infected with recombinant AcNPV.** Cells infected with AcNPV-interferon expression vectors underwent morphological changes that are typical of  $O^-$  viral infections (30). The entire infection process, including viral protein synthesis, viral assembly, and partial

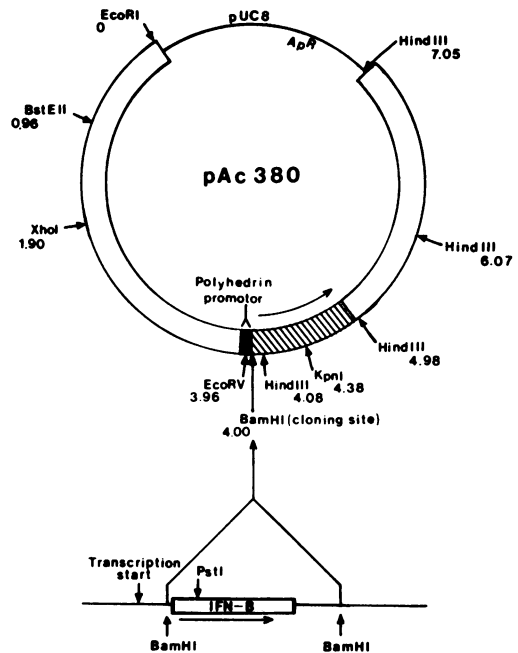


FIG. 3. AcNPV transfer vector pAc380 and IFN- $\beta$ . Arrows mark the approximate location of the polyhedrin mRNA coding sequences, and the positions of certain restriction sites are indicated in kilobase pairs. The *Bam*HI cloning site at 4.00 is the position at which a deletion from +175 to -59 (Fig. 2) was introduced and replaced by a *Bam*HI octanucleotide linker. *Bam*HI linkers were added to a 767-base-pair *Hinc*II IFN- $\beta$  fragment and then cloned into the *Bam*HI site in pAc380, producing the recombinant transfer vector pAc380-IFN- $\beta$ . IFN- $\beta$  protein-coding sequences are indicated by the rectangle, and the long arrow marks the direction of transcription.

cell lysis, was complete by ca. 72 h p.i. There was a marked reduction in protein synthesis between 50 and 60 h p.i., and cell lysis was first detected at about 50 h p.i. The titer of recombinant AcNPV-infected cells reached a maximum of ca.  $5 \times 10^8$  PFU/ml of medium, which is typical of AcNPV-infected cells. Thus, the insertion of interferon DNA into the polyhedrin gene had no major effect on the replication of these recombinant viruses.

To determine whether the AcNPV polyhedrin gene sequences could promote expression of IFN- $\beta$ , the level of interferon activity was measured in *S. frugiperda* cells infected with each of the AcNPV expression vectors. Because interferon is normally secreted, media from infected cells were also tested for interferon activity. We used the virus plaque-reduction assay (18) in WISH cells challenged with vesicular stomatitis virus to determine interferon activity. If virus particles were removed by centrifugation, no interferon activity was measured in medium

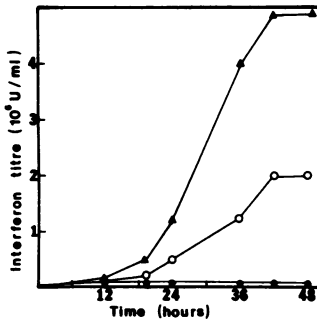


FIG. 4. Production of interferon activity in infected cells *S. frugiperda* cells were infected with AcNPV (●), Ac360-IFN- $\beta$  (○), or Ac380-IFN- $\beta$  (▲). The interferon activity in the medium was measured at 0, 12, 20, 24, 36, 42, and 48 h p.i. as described for Fig. 2.

from wild-type AcNPV-infected cells. However, if AcNPV-containing media were used during interferon assays 1,000 to 3,000 U of interferon per ml was produced, indicating that AcNPV virions apparently can induce the expression of endogenous interferon in WISH cells. Because many species of enveloped viruses are known to induce interferon production in human cells (39), these results were expected. To avoid this effect, all subsequent samples were centrifuged before testing.

Significant levels of interferon were detected by 48 h p.i. in cells infected with all of the expression vectors. Ac380-IFN- $\beta$ , in which interferon was inserted about 19 bases downstream from the polyhedrin TATA box sequences, produced the highest levels (Fig. 2). Very high levels were also produced by Ac360-IFN- $\beta$ , in which interferon was located at +34 and in the same translational reading frame as polyhedrin. In contrast, very low levels of activity were produced in cells infected with Ac101-IFN- $\beta$  (Fig. 2), in which interferon was in the same reading frame as polyhedrin but located much further downstream at +175. Somewhat unexpected were the moderate levels of interferon activity produced by cells infected with Ac311-IFN- $\beta$  (Fig. 2), in which interferon was inserted at +35 and consequently not in the correct reading frame for polyhedrin. Described later is an examination of proteins produced in infected cells, which helps to explain these results.

The kinetics of interferon induction were examined in *S. frugiperda* cells infected with the expression vectors Ac360-IFN- $\beta$  and Ac380-IFN- $\beta$ . Samples of the medium were taken from 0 to 48 h p.i. and stored at  $-80^{\circ}\text{C}$  until assayed for interferon. The medium from Ac380-IFN- $\beta$  infected cells had about 10,000 IU of interferon per ml at 12 h p.i., and this level increased to a

maximum of nearly  $5 \times 10^6$  IU/ml by 42 h p.i. (Fig. 4). A similar pattern of induction was observed in Ac360-IFN- $\beta$ -infected cells (Fig. 4). The synthesis of polyhedrin in AcNPV-infected cells is known to follow a similar temporal pattern of expression (see reference 33 and references therein).

**Effects of IFN- $\beta$  on *S. frugiperda* cells.** A potential limitation in using animal virus expression vectors for interferons (7) is that interferon itself can impose restraints upon the level of expression by interfering with the replication of the virus vector. The high levels of IFN- $\beta$  produced in *S. frugiperda* cells, using AcNPV as a vector, and the normal titer of virus that was produced suggested that interferon does not affect AcNPV replication. To examine whether human IFN- $\beta$  induced an antiviral state in *S. frugiperda* cells, the following experiment was conducted. Cells ( $2 \times 10^6$ ) were treated for 12 h with, per ml, up to  $5 \times 10^6$  IU of interferon produced in Ac380-IFN- $\beta$ -infected cells or  $5 \times 10^3$  IU of an international standard of IFN- $\beta$ , and the treated cells were infected with 100 PFU of AcNPV or Ac380-IFN- $\beta$ . Exposure of the cells to human interferon had no measurable effect on the number of virus plaques that developed.

**Protein synthesis in cells infected with AcNPV expression vectors.** In the expression vector Ac380-IFN- $\beta$  the nucleotides between  $-59$  and  $+175$  were deleted and replaced by an IFN- $\beta$  gene fragment containing the interferon ATG translational start signal preceded by three bases and a synthetic *Bam*HI linker (Fig. 2 and 3). This placed the interferon gene ATG protein start signal approximately the same number of bases downstream from the polyhedrin gene TATA box as the polyhedrin transcriptional start site, leaving little, if any, nontranslated leader at the 5' end of the predicted mRNA for this hybrid gene. High levels of interferon activity were measured in the medium of Ac380-IFN- $\beta$ -infected cells, indicating that the polyhedrin promoter efficiently directed the transcription of this gene. At 30 and 48 h p.i., two polypeptides of 17,000 (17K) and 20.5K molecular weight were being made in Ac380-IFN- $\beta$  but not in AcNPV-infected cells (Fig. 5A and B). The sizes of non-glycosylated and glycosylated human IFN- $\beta$  proteins are reported to be comparable to the 17K and 20.5K polypeptides, respectively (3, 15). At 30 h p.i., the 17K polypeptide was being made in Ac360-IFN- $\beta$ -infected cells (Fig. 5A; see Fig. 7), and by 48 h p.i., both 17K and 20.5K polypeptides were detected (Fig. 5B). Only the 17K polypeptide could be detected in Ac311-IFN- $\beta$ -infected cells at 30 and 48 h p.i. (data not shown). An abundantly produced 23.5K protein was observed in Ac360-IFN- $\beta$ -infected cells

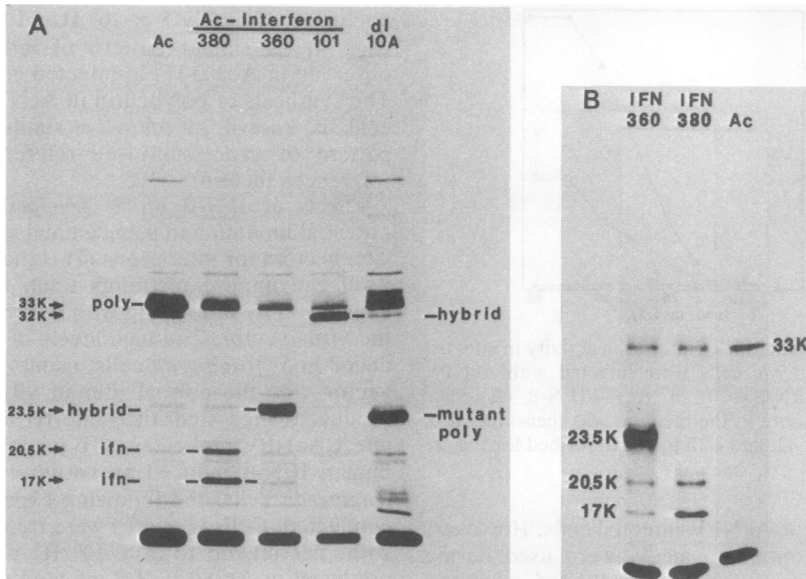


FIG. 5. Recombinant interferon produced in cells at (A) 30 or (B) 48 h p.i. (A) *S. frugiperda* cells were infected with AcNPV (Ac), Ac380-IFN- $\beta$ , Ac360-IFN- $\beta$ , Ac101-IFN- $\beta$ , and *d/10A* (a deletion mutant of AcNPV that produces a truncated polyhedrin protein) and then labeled for 30 min with [ $^3$ H]leucine at 30 h p.i. (B) *S. frugiperda* cells were infected with AcNPV (Ac), Ac380-IFN- $\beta$ , and Ac360-IFN- $\beta$  and then labeled for 30 min with [ $^3$ H]leucine at 48 h p.i. Total cell lysates were electrophoresed on a sodium dodecyl sulfate-12% polyacrylamide gel; the gel was impregnated with En $^3$ Hance (New England Nuclear Corp., Boston, Mass.) and exposed to X-ray film for 2 days at  $-80^\circ\text{C}$ . The molecular weights ( $\times 10^3$ ) of certain polypeptides are indicated.

(Fig. 5A and B) and has the size expected for a hybrid protein consisting of the entire interferon protein, including the 21 amino acid signal peptide plus an additional 14 amino acids derived from the first 10 codons of the polyhedrin gene and the *Bam*HI linker sequences (Fig. 1 and 2).

In the expression vector Ac101-IFN- $\beta$ , the interferon fragment was inserted into the natural AcNPV *Bam*HI site at +175 in the same translational reading frame as polyhedrin. A putative hybrid 32K polypeptide was made in cells infected with this expression vector (Fig. 5). The size of the protein was as predicted from the DNA sequence.

**Immunological identification of recombinant products.** The 17K and 20.5K proteins being made in Ac380-IFN- $\beta$ - and, to a lesser extent, Ac360-IFN- $\beta$ -infected cells reacted with human IFN- $\beta$  monoclonal antibody (Fig. 6C). The autoradiogram shown in Fig. 6 is a 6-h exposure, and the reaction of IFN- $\beta$  antibody to the 20.5K protein was more obvious with longer exposure times. The reduced reaction of this antibody to the 20.5K protein as compared with that to the 17K protein was in part due to the fact that 17K accumulates to higher levels in cells than does 20.5K (data not shown). In addition, the antibody may be reacting to an epitope on the 17K polypeptide that is partially masked by, for example, glycosylation of the 20.5K polypep-

ptide. The putative hybrid 23.5K and 32K proteins also reacted with IFN- $\beta$  antibody (Fig. 6C). Polyclonal antibody to polyhedrin recognized the 10 amino acids of the 23.5K protein and the 57 amino acids of the 32K protein that would be predicted from the DNA sequence to be present at the N-terminal ends of the hybrid proteins (Fig. 6B).

**Glycosylation of IFN- $\beta$  in infected cells.** Tunicamycin is a specific inhibitor of glycosylation which prevents the assembly of glycoproteins (38). The inhibitor has been used to produce nonglycosylated IFN- $\beta$  (42) and is effective in AcNPV-infected *S. frugiperda* cells (36). In the presence of tunicamycin, a 64K AcNPV glycoprotein (6) was missing and a new protein of 58K was present (Fig. 7), indicating that the concentration of inhibitor used in this experiment (5  $\mu\text{g}/\text{ml}$ ) was sufficient to block glycosylation of this protein. Tunicamycin had little effect on the synthesis of the 17K IFN- $\beta$  or the 23K and 32K hybrid proteins in Ac360-IFN- $\beta$ - and Ac101-IFN- $\beta$ -infected cells (Fig. 7). However, no 20.5K IFN- $\beta$  was produced in the presence of tunicamycin in cells infected with Ac360-IFN- $\beta$  (Fig. 7) or Ac380-IFN- $\beta$  (data not shown).

To further demonstrate that 20.5K IFN- $\beta$  was glycosylated, Ac380-IFN- $\beta$ -infected cells were labeled late in infection with [ $^3$ H]mannose. The 20.5K IFN- $\beta$  and three additional proteins were

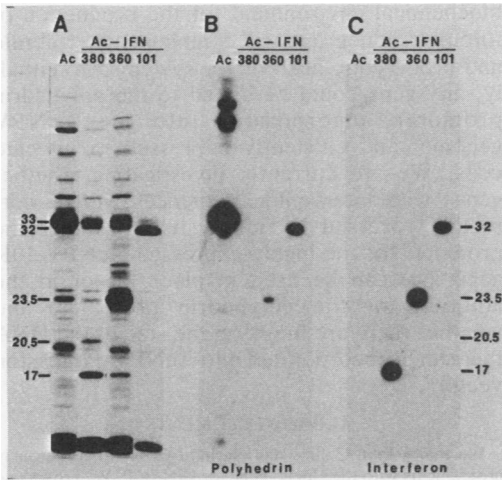


FIG. 6. Immunological identification of interferon and polyhedrin-interferon hybrid proteins. At 40 h p.i., total-cell lysates from AcNPV (Ac-), Ac380-IFN- $\beta$ -, Ac360-IFN- $\beta$ -, and Ac101-IFN- $\beta$ - infected cells were electrophoresed on a sodium dodecyl sulfate-12% polyacrylamide gel, transferred by diffusion to two nitrocellulose filters, and incubated with either polyhedrin antiserum or IFN- $\beta$  monoclonal antibody as described previously (32). Specifically bound antibody was detected with  $^{125}\text{I}$ -labeled protein A, and the filters were exposed to X-ray film at  $-80^\circ\text{C}$  for 6 h with the aid of an intensifying screen. (A) Autoradiogram of infected cells labeled with  $^3\text{H}$ leucine at 40 h p.i. as described for Fig. 5. The molecular weights ( $\times 10^3$ ) of certain proteins are marked.

the major mannose-containing glycoproteins labeled in Ac380-IFN- $\beta$ -infected cells (Fig. 8). A summary of the 17K non-glycosylated and 20.5K glycosylated IFN- $\beta$  and the polyhedrin-IFN- $\beta$  hybrid proteins produced by the various expression vectors is given in Fig. 2.

DISCUSSION

We have demonstrated that exceptionally high levels of interferon were produced in cells infected with a recombinant AcNPV expression vector in which the gene for IFN- $\beta$  was placed under the transcriptional control of the polyhedrin promoter. Based on the specific activity of purified interferon (14), we estimate that ca. 10  $\mu\text{g}$  of interferon was secreted by  $10^6$  *S. frugiperda* cells infected with Ac380-IFN- $\beta$ . In Ac380-IFN- $\beta$ , the coding sequences for interferon were linked to the polyhedrin promoter at -59 (ca. 19 bases downstream from the canonical TATA sequences), suggesting that (i) polyhedrin gene sequences upstream from -59 were primarily responsible for the regulation of transcription and (ii) the 58-base nontranslated 5'-

leader sequence of polyhedrin was not absolutely required for efficient expression.

Cleavage of the signal peptide of pre-IFN- $\beta$  is thought to be essential for producing biologically active, mature IFN- $\beta$  (3, 37). Therefore, the polyhedrin-IFN- $\beta$  hybrid proteins produced by the vectors Ac360-IFN- $\beta$  and Ac101-IFN- $\beta$ , which almost certainly still have the pre-IFN- $\beta$  signal peptide as well as additional polyhedrin sequences, were most likely not biologically active. Much of the mature IFN- $\beta$  produced by Ac360-IFN- $\beta$  was probably a result of the removal of the signal peptide from some of the 23.5K hybrid gene product, in spite of the fact that the 21-amino acid signal sequence was an additional 14 amino acids from the N-terminal end of the protein. Sequence analysis of IFN- $\beta$  and the hybrid proteins will be needed to confirm these speculations. Mature IFN- $\beta$  may have also been produced by Ac360-IFN- $\beta$  by another mechanism. It can be predicted from the scanning model for eucaryotic translation initiation (16) that the polyhedrin protein start sequence AUAUGC is less favorable for initia-

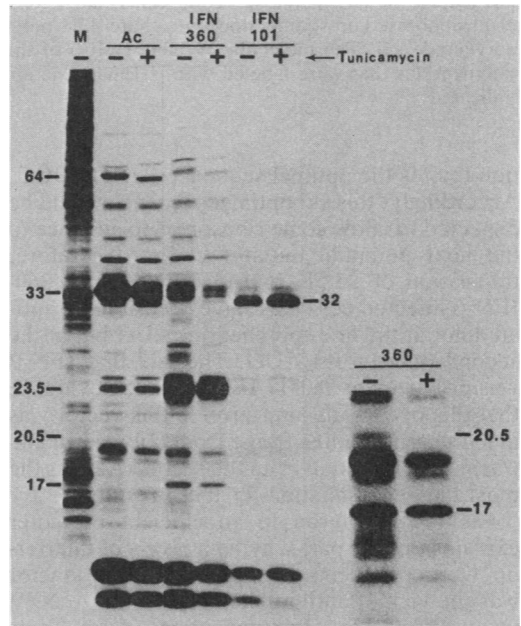


FIG. 7. Effects of tunicamycin on the synthesis of interferon. At 30 h p.i. mock (M)-, AcNPV (Ac)-, Ac360-IFN- $\beta$ -, and Ac101-IFN- $\beta$ - infected cells were labeled with  $^3\text{H}$ leucine for 30 min in the presence or absence of 5  $\mu\text{g}$  of tunicamycin per ml. Infected cells were pretreated for 10 h in medium plus 5  $\mu\text{g}$  of tunicamycin per ml before being labeled in the presence of the inhibitor. A longer exposure of Ac360-IFN- $\beta$ -labeled proteins is shown in the right panel. The molecular weights ( $\times 10^3$ ) of certain proteins are marked.



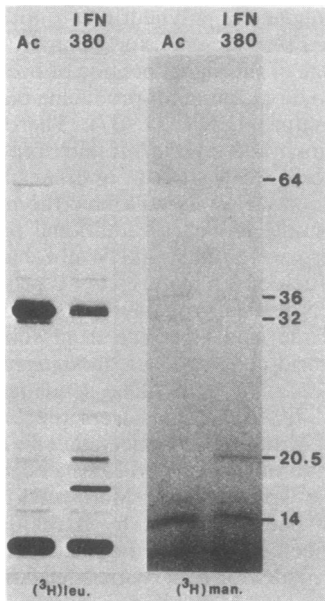


FIG. 8. Incorporation of [ $^3\text{H}$ ]mannose into recombinant interferon. AcNPV (Ac)- and Ac380-IFN- $\beta$ -infected cells were labeled for 1 h at 40 h p.i. with [ $^3\text{H}$ ]leucine or [ $^3\text{H}$ ]mannose. Total cell lysates were electrophoresed on sodium dodecyl sulfate-12% polyacrylamide gel. The molecular weights ( $\times 10^3$ ) of the polypeptides that were labeled with [ $^3\text{H}$ ]mannose are indicated.

tion than is the optimal sequence PuNNAUGG. Accordingly, this suboptimal sequence would be expected to allow some ribosomes to advance to the next potential initiator codon. Therefore, translation of 23.5K hybrid mRNA in Ac360-IFN- $\beta$  infected cells may have occasionally initiated not at the first (polyhedrin) AUG but at the second (interferon) AUG. The fact that IFN- $\beta$  was produced by Ac311-IFN- $\beta$  is good evidence that this occurs, as interferon in this vector was in a different reading frame from polyhedrin and translation initiation from the second AUG is the most likely explanation for these results.

Linking interferon to an efficient promoter explains only in part why high levels of interferon were produced. Another important factor was the lack of antiviral activity on the AcNPV vector itself. The millions of interferon units produced per milliliter in our system had no measurable effect on virus yields, whereas only 100 U of interferon per ml reduces the yield of simian virus 40 by up to 80% (7), limiting the use of this and similarly affected vertebrate animal viruses as vectors for interferon.

AcNPV and other baculoviruses should prove to be important vectors for the production of cloned gene products in insect cells or organisms. The invertebrate cell will provide a unique

biochemical environment for the production of foreign products and will complement vertebrate and procaryotic host-vector systems. Potentially, any gene could be linked to the polyhedrin promoter, incorporated into the AcNPV genome, and efficiently expressed in infected cells. We are currently investigating whether genes with intervening sequences will be correctly processed in this system, whether the promoter for the highly expressed AcNPV 10K gene (34) can be used in place of, or in the addition to, the polyhedrin promoter, and whether there are limits on the size of the DNA that can be incorporated into AcNPV expression vectors.

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#### ADDENDUM IN PROOF

After this report was submitted for review a study by Remaut et al. (E. Remaut, P. Stanssens, and W. Fiers, *Nucleic Acids Res.* **14**:4677-4688, 1983) was published which described the synthesis of mature IFN- $\beta$  under the transcriptional control of a phage lambda promoter in *E. coli* cells at a level similar to that which was reported here.

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