Hyperproduction of Polyhedrin–IGF II Fusion Protein in Silkworm Larvae Infected with Recombinant Bombyx mori Nuclear Polyhedrosis Virus

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SUMMARY

A gene coding for insulin-like growth factor II (IGF II) was constructed from 16 oligodeoxynucleotides synthesized chemically and cloned into EcoRI–SalI sites of pBR322. In this gene an ATG codon for methionine was introduced for cleavage by CNBr at the beginning of mature IGF II. For expressing foreign genes, a new host–vector system, with Bombyx mori silkworm larvae as the host and B. mori nuclear polyhedrosis virus (BmNPV) as the vector, has been developed. BmNPV genomic DNA codes polyhedrin which is a major protein of inclusion bodies and is mass-produced in infected silkworm larvae. We employed this polyhedrin production system to obtain a large yield of a foreign gene product. The coding region of the carboxy-terminal half of polyhedrin was removed and the remainder was ligated with the IGF II gene in phase to create a fusion protein gene consisting of the coding region of the amino-terminal half of polyhedrin and the IGF II gene. This fusion protein gene was combined in a plasmid with the promoter and 5' and 3' flanking regions of the polyhedrin gene. The resulting plasmid and the wild-type BmNPV genomic DNA were cotransfected into BM-N cells, and a recombinant virus was isolated by the limiting dilution method. The silkworm larvae infected with the recombinant virus produced 3-6 mg of the fusion protein per larva and the infected BM-N cells produced 0-3 mg per ml of culture. IGF II was released from the fusion protein produced by BM-N cells infected with the recombinant virus by CNBr treatment, purified by extraction with guanidine–HCl, column chromatography and HPLC and the correct amino-terminal amino acid sequence confirmed.

INTRODUCTION

In order to express foreign genes, new host–vector systems employing insect viruses and insect cell lines or larval bodies have recently been developed. Recombinant vaccinia virus was created to produce Sindbis virus proteins in Aedes albopictus mosquito cell lines (Franke & Hruby, 1985). The baculoviruses have been used as vectors in insect cell lines or intact larvae. Interferon-β (IFN-β), β-galactosidase, interleukin 2 and human c-my c protein have been produced by established Spodoptera frugiperda cell lines infected with recombinant Autographa californica nuclear polyhedrosis viruses harbouring the genes for these proteins (Smith et al., 1983, 1985; Pennock et al., 1984; Miyamoto et al., 1985). IFN-α was secreted from Bombyx mori silkworm cultured cells and larvae infected with recombinant B. mori nuclear polyhedrosis virus (BmNPV) into culture fluid and larval haemolymph, respectively (Maeda et al., 1984, 1985). BmNPV, a member of the family Baculoviridae, has a double-stranded circular DNA genome over 100 kbp in length which contains the polyhedrin gene (735 bp). Polyhedrin, the major protein of inclusion bodies in which mature virus particles are embedded, is mass-produced in
infected cell lines and larvae in the form of small aggregates, and its yield exceeds 10 mg per infected larva. However the yield of IFN-α was less than that of polyhedrin.

In order to obtain large amounts of foreign proteins, the respective genes have been expressed as fusion products, especially for relatively small proteins in mammalian cells (Nielsen et al., 1983) and Escherichia coli (Buell et al., 1985; Peters et al., 1985). In order to obtain a large amount of insulin-like growth factor II (IGF II), we constructed the IGF II gene from chemically synthesized oligodeoxynucleotides and expressed it as a polyhedrin-IGF II fusion protein in both larvae and a B. mori cell line (BM-N) using recombinant BmNPV. IGF II was isolated from the fusion protein by CNBr cleavage followed by chromatography.

METHODS

Enzymes and general manipulations. Enzymes were purchased from Takara Shuzo (Kyoto, Japan), Nippon Gene, Bethesda Research Laboratories and New England Biolabs. Most enzyme reactions proceeded under the conditions recommended by the suppliers. Transformation of E. coli MC1061 or HB101 was carried out according to Bolivar & Backman (1979) using RbCl. Colony hybridizations using synthetic oligodeoxynucleotides were performed as in Wallace et al. (1981a).

Silkworm rearing. Silkworms at the fifth instar were provided by Kyodo Shiryo Co. Ltd., where they had been reared after hatching at 29 °C in the dark with artificial diets. After infection, silkworms were reared at 28 °C in small plastic containers, at five silkworms per container. Artificial diets mainly consisted of dried mulberry leaf powder, defatted soybean meal and cellulose (Ito & Kobayashi, 1978).

Chemical synthesis of oligodeoxynucleotides. Sixteen oligodeoxynucleotides (18-mers to 35-mers) were synthesized by the solid-phase phosphate–phosphotriester method on a polystyrene support (Crea et al., 1978; Kierzek et al., 1981). Oligomers were deprotected with concentrated ammonia water and purified by gel filtration using a Sephadex G-50 and reversed-phase HPLC, followed by removal of the dimethoxytryptyl residue at the 5' terminus with 80% acetic acid and repurification through HPLC.

Insertion of the fusion protein gene into the BmNPV genome. A solution comprising 2.5 ml of 50 mM-HEPES buffer pH 7.1, 0.28 M-NaCl, 0.7 mM-Na₂CO₃ and 0.7 mM NaHPO₄ was added to the DNA mixture (2.5 ml of 0.24 M-CaCl₂ containing 10 µg of BmNPV DNA and 50 µg of plasmid DNA) to form a fine precipitate. About 0.5 ml of this precipitate was added to 2 × 10⁶ BM-N cells in a flask containing 4 ml of TC-10 medium supplemented with 10% foetal calf serum (Gardiner & Stockdale, 1975). After incubation for 20 h at 27 °C the culture fluid was replaced with fresh medium and incubated for a further 6 days. After centrifugation at 2000 r.p.m. for 10 min, the supernatant, containing a mixture of recombinant and wild-type virus, was diluted with fresh medium to adjust the virus concentration to 5 p.f.u. per ml and an equal volume of suspended BM-N cells (1.5 × 10⁶ cells per ml) was added. Two-hundred µl of this cell–virus suspension was poured into each well of a microwell plate and incubated for 7 days. The viruses that could not produce normal inclusion bodies were isolated and repurified. The recombinant virus obtained was designated vFIGF II 120; its DNA was analyzed by Southern blot hybridization (Perbal, 1984) to confirm the insertion of the fusion gene.

Quantitative analysis of the fusion protein. The yield of the fusion protein was determined by quantitative analysis of polyacrylamide gels stained with Coomassie Brilliant Blue (Manabe & Okuyama, 1983). The quantity of protein was calculated by comparison with the density of standards of known molecular weights.

Recovery of IGF II from the fusion protein. BM-N cells infected with vFIGF II 120 were harvested by centrifugation at 1000 r.p.m. for 10 min on the fourth day after infection and disrupted by freezing in a dry ice/acetonitrile bath followed by thawing in a 37 °C water bath three times. After centrifugation at 3000 r.p.m. for 10 min, the sediment was washed with 3 M-urea solution and then dissolved in 6 M-guanidine-HCl solution and centrifuged at 3000 r.p.m. for a further 10 min to remove the residue. The crude fusion protein was precipitated by dialysis against water and recovered by centrifugation at 3000 r.p.m. for 10 min. The protein was dissolved in 70% formic acid and subjected to treatment with CNBr at a concentration of 10 mg per ml for 24 h at room temperature followed by concentration under vacuum. The crude IGF II was obtained through ion-exchange column chromatography with a pyridine–acetate gradient as described by Rinderknecht & Humbel (1976). IGF II was further purified using HPLC with an acetonitrile gradient in 0-1% trifluoroacetic acid.

RESULTS AND DISCUSSION

Construction of the IGF II gene

IGF II belongs to the somatomedin group which have growth-promoting effects on mammalian cells, and it has an essential role in foetal growth (Czech, 1982; Adams et al., 1983). The primary structure was determined as a 67 amino acid polypeptide (Rinderknecht & Humbel, 1978). Figure 1 shows the amino acid sequence of IGF II and the DNA sequence of the
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**Fig. 1.** The amino acid sequence of IGF II and the DNA sequence of its synthesized gene. IG 1 to 16, synthesized oligodeoxynucleotides. The first amino acid, methionine, is positioned for cleavage by CNBr, and alanine is the amino-terminal residue of mature IGF II.

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**Fig. 2.** Construction of the IGF II gene. Each oligomer except IG 1, 8, 9 and 16 was phosphorylated using T4 polynucleotide kinase. A mixture of 200 pmol each of IG 1, 2, 3 and 4 was heated at 90 °C and cooled slowly to 4 °C. The ligation reaction was carried out at 4 °C for 18 h using T4 DNA ligase and followed by recovery of fragment A by PAGE on an 8 % gel. Fragments B, C and D were purified in the same manner. Fragment L was purified from the mixture of A and B by PAGE preceded by a ligation reaction using T4 DNA ligase. Fragment R was obtained similarly. In the final step of the construction, fragments R, L and pBR322 digested with EcoRI and SalI participated in a three-part ligation and the resulting reaction product, pIGF II 002, was used to transform E. coli MC1061.

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synthetic gene. Sixteen chemically synthesized oligodeoxynucleotides (IG 1 to 16) are also shown in the same figure. The amino-terminal residue of mature IGF II is alanine and the first amino acid, the methionine encoded by the ATG of this gene, permitted CNBr cleavage of the fusion protein to release IGF II. The construction of the gene from individual oligomers is illustrated in Fig. 2. Restriction sites for EcoRI and SalI were added at each end of the gene to
Fig. 3. Construction of the fusion protein gene. A partial restriction map of BmNPV around the polyhedrin gene (represented by hatched blocks) and its promoter (represented by dotted blocks) is shown on the top. Plasmid p9H 18, containing the polyhedrin gene, was treated with EcoRI and digested with exonuclease Bal31 to partially delete the carboxy-terminal region of the gene, followed by digestion with HindIII. The fragment including the partially deleted gene was subcloned into pUC9 pretreated with HindIII and SmaI. After pIGF 002 had been partially digested with SalI, DNA polymerase I Klenow fragment treatment was used to create blunt termini, followed by HaeIII digestion. The IGF II fragment was recovered by PAGE on an 8% gel and inserted into the SalI site of pBM 030 to construct pIGF II 030. This was then partially digested with EcoRI and Scal. The fragment containing the IGF II gene and the 3' flanking region of the polyhedrin gene was purified by agarose gel electrophoresis. From p9B 120 the EcoRI–Scal fragment, on which the deleted polyhedrin gene and 5' flanking region including promoter were carried, was isolated through agarose gel electrophoresis and ligated with pIGF II 030 using T4 DNA ligase to obtain pFIGF II 120.

facilitate insertion into pBR322, and the XbaI site in the middle of the gene was designed to make the confirmation of the DNA sequence more convenient. The DNA sequence of the gene in pIGF 002 was confirmed by the Maxam–Gilbert method (Maxam & Gilbert, 1980).
Polyhedrin

ATG GACC TAA (738 bp)

Fusion protein

ATG ATG TAG (549 bp)

Fig. 4. Diagram of the fusion protein gene. The numbers above the polyhedrin gene indicate nucleotide positions. The amino acids and the DNA sequence around the fusion site are also shown. The fusion protein consists of 182 amino acids: 111 from polyhedrin, three from the DNA linker, 68 from IGF II, including methionine preceding mature IGF II.

Construction of the fusion protein gene

In order to obtain a partially deleted polyhedrin gene which lacked a carboxy-terminal region at an appropriate site, Bal31 digestion of p9H18 followed by subcloning into pUC9 was employed as shown in Fig. 3. Of the plasmids obtained in this digestion, p9B120 carried a partially deleted polyhedrin gene which, downstream, lacked position 338 of the gene, and this contained 46% of the 735 bp polyhedrin gene. This plasmid, which was used to construct the fusion protein gene, harbourd the promoter and the 5' flanking region of the polyhedrin gene and the deleted gene, but did not have a 3' flanking region.

The fragment containing the IGF II gene from pIGF002 was inserted into the SmaI site of pBM030, which harboured the promoter, 5' and 3' flanking regions of the polyhedrin gene and a polylinker in place of the polyhedrin gene. The IGF II gene attached to the 3' flanking region from this plasmid was ligated to the deleted polyhedrin gene from p9B120 in phase in order to create the fusion protein gene, and the resulting plasmid was designated pFIGFII120. As shown in Fig. 4, in this fusion protein the amino-terminal half of polyhedrin and IGF II were connected by three additional amino acids, Arg, Glu and Phe, originating from linker DNA, and it was planned that IGF II should be preceded by methionine so that it could be cleaved by CNBr to isolate IGF II. The nucleotide sequence around the fusion site was confirmed by the dideoxy sequencing method (Hattori & Sakaki, 1986).

Isolation of the recombinant virus

Insertion of the fusion protein gene into the BmNPV genome using pFIGFII120 was carried out according to Maeda et al. (1985). BM-N cells were cotransfected with the plasmid and viral genomic DNA in a fine precipitate of calcium phosphate to generate recombinant virus by genetic recombination which must take place in the cells. In this recombination, the 5' and 3' flanking regions of the polyhedrin gene situated at each side of the fusion protein gene in pFIGFII120 provided homologous regions to ensure that recombination would occur as expected at the site of the polyhedrin gene. Recombinant virus clones were obtained by the limiting dilution method as described in Methods. Genomic DNA extracted from the recombinant virus named vFIGFII120, which lacked the ability to produce normal polyhedral inclusion bodies, was analysed by Southern blot hybridization using 32P-labelled synthetic oligodeoxynucleotides as probes (Wallace et al., 1981b) to confirm the presence of the fusion protein gene. Replacement of the polyhedrin gene with the fusion protein gene is demonstrated in Fig. 5, which shows that the 3-7 kbp fusion protein gene fragment is 0.2 kbp smaller than the polyhedrin gene fragment, and that it hybridizes with pr-05 and IG 3 but not with pr-06. This result indicates that vFIGFII120 has the fusion protein gene at the exact site from which the polyhedrin gene had been lost.
Fig. 5. Southern blot hybridization analysis of recombinant virus. Viral DNA from wild-type phage T3 (lane 1) and recombinant virus vFIGF II 120 (lane 2) was digested with *HindIII* and separated in a 1% agarose gel (a). After transfer to a nitrocellulose filter, hybridizations were carried out using the synthetic oligodeoxynucleotides pr-06 (b) and pr-05 (c) which were complementary to the 3' and 5' ends, respectively, of the polyhedrin gene, and IG 3 (d), part of the IGF II gene, as probes.

**Production of the fusion protein**

The fusion protein whose production was carried out using infected silkworm larvae of the fifth instar or infected BM-N cells in a flask was analysed by SDS-PAGE and with a densitometer as shown in Fig. 6. The reason why the apparent size of the fusion protein (about 24K) is greater than the calculated size (21.4K) is not clear. On the fourth day the production of the fusion protein reached a maximum of 3.6 mg per infected larval body and of 0.3 mg per ml of infected cell culture. In the cell culture, the number of cells did not significantly increase after infection. Lysis of cells occurred on the fifth day, which led to difficulties in recovering the product from the flask, resulting in a decrease of the yield after the fifth day.

BM-N cells infected with vFIGF II 120 were collected and disrupted by freeze–thawing three times. After centrifugation at 3000 r.p.m. for 10 min the fusion protein could not be detected in either the supernatant or the culture fluid but was present in the SDS extract from the sediment fraction analysed by SDS–PAGE (data not shown). Furthermore, this fusion protein was recovered as a precipitate from guanidine–HCl solution by dialysis against water. These data suggest that the fusion protein, like polyhedrin, is insoluble in water at neutral pH. The hydrophobic character was retained in the amino-terminal half of polyhedrin and even in the fusion protein of which IGF II accounts for nearly 40%. This results in its precipitation within the cells, with consequent resistance to proteolysis, and therefore a large amount of it may be allowed to accumulate as demonstrated by Jones & Brownlee (1985) for the accumulation of a precipitated fusion protein consisting of influenza virus N protein with Cro peptide within *E. coli* cells.
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Fig. 6. Production of the fusion protein in larvae and cultured cells. Fifty μl (1 x 10⁷ p.f.u.) of viral suspension was injected into the body cavity of the larvae at an early stage of the fifth instar. Every day after infection, five larvae were transferred to a 50 ml tube and frozen at -80 °C. After 15 ml of 12.5% SDS solution had been added, the bodies were crushed, sonicated for 3 min on ice, sedimented at 2000 r.p.m. for 20 min and then 1 μl of supernatant was analysed by SDS-PAGE on a 10 to 20% gradient gel. A monolayer of BM-N cells (2 x 10⁶ cells) was infected with 5 x 10⁷ p.f.u. virus in a flask containing 4.5 ml of TC-10 medium supplemented with 10% foetal calf serum and incubated for 30 min. Replacement of the fluid with fresh medium was followed by further incubation. Cells were harvested at 24 h intervals by centrifugation at 2000 r.p.m. for 10 min and were suspended in 450 μl of water. One μl of this suspension was mixed with 4 μl SDS solution and analysed by SDS-PAGE on a 10 to 20% gradient gel. (a) Production of the fusion protein (fus) and polyhedrin (pol) on the fourth day after infection with vFIGF II 120 (lanes 3 and 6) or wild-type virus T3 (lanes 2 and 5), or without infection (lanes 1 and 4), by larvae (lanes 1 to 3) or BM-N cells (lanes 4 to 6). Mol. wt. values are x 10⁻³.

(b) Time course of fusion protein production by larvae (▲) and BM-N cells (○) infected by vFIGF II 120.

Fig. 7. SDS-PAGE of IGF II isolated from the fusion protein produced by BM-N cells on a 20% gel. Lane 1, mol. wt. markers (x 10⁻³), lane 2, IGF II.

Recovery of IGF II from the fusion protein

The fusion protein produced by the infected BM-N cells was pure enough for cleavage by CNBr after purification by extraction with 6 M-guanidine–HCl and precipitation by dialysis. Purification of IGF II released from the fusion protein was achieved through ion-exchange chromatography and HPLC (Fig. 7). The IGF II obtained in this study was subjected to gas-phase Edman degradation in an Applied Biosystems protein sequencer and the eight amino-terminal amino acids were confirmed.

In this study, IGF II was mass-produced in silkworm larvae in the form of a fusion protein. Breeding a large number of silkworms requires no difficult procedures or special costly equipment and inexpensive artificial feed may be used throughout the year. Large amounts of the fusion protein can therefore be obtained easily and at low cost. Methionine was inserted into this fusion protein at the beginning of the IGF II so as to make recovery by CNBr treatment possible. Attempts to recover IGF II from the fusion protein produced in silkworm larvae are now in progress.

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