Protein synthesis in pupae of the silkworm *Bombyx mori* after infection with nuclear polyhedrosis virus: resistance to viral infection acquired during pupal period

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Protein synthesis has been studied in pupae of the silkworm *Bombyx mori* (Bm) infected with nuclear polyhedrosis virus (BmNPV) at various stages of the pupal period. Nascent proteins were labelled by injection of $\text{[}^{35}\text{S}\text{]}\text{methionine}$ into pupae and then analysed by SDS–PAGE. Temporal regulation of synthesis of infected cell-specific proteins (ICSPs) in pupae was demonstrated by electrophoretic analysis of the proteins labelled at different times post-infection (p.i.). The rate of ICSP synthesis reached a maximum at 4 to 5 days p.i., exceeding the rate of synthesis of cellular proteins in uninfected pupae by about twofold. The viral proteins pl0 and polyhedrin were the most abundant products synthesized late in the infection. Both proteins were found to be associated with the nuclear matrix after fractionation of nuclei from infected pupae. Two virus-induced phosphoproteins, pp35 and ppB, were found to be the major acceptors of labelled phosphate from $[\gamma-\text{32P}]\text{ATP}$ during *in vitro* phosphorylation of proteins in pupal homogenates, nuclei and nuclear extracts. These proteins had electrophoretic mobilities comparable to those of structural phosphoproteins of BmNPV virions with $M_r$s of 35K and 11K to 16K, respectively. The latter polypeptide was identified as the major DNA-binding protein of the virus. The susceptibility of silkworms to BmNPV decreased markedly during the pupal period. Following injection of BmNPV all young pupae acquired polyhedrosis and finally died whereas most of the older pupae did not exhibit disease and completed metamorphosis normally. Moreover, the later in the pupal period the silkworms were infected, the lower the production of polyhedrin in diseased pupae.

Introduction

*Bombyx mori* nuclear polyhedrosis virus (BmNPV) is a member of the subfamily Eubaculovirinae of the family Baculoviridae (Francki *et al.*, 1991). It contains a circular dsDNA genome of about 130 kb (Iatrou *et al.*, 1985; Maeda & Majima, 1990). Upon infection of insect cells the nuclear polyhedrosis viruses (NPVs) induce temporally regulated synthesis of infected cell-specific proteins (ICSPs). On the basis of the requirement for *de novo* protein synthesis for viral DNA replication, expression of the NPV genome can be divided into four phases: immediate early, delayed early, late and very late (Miller *et al.*, 1983a; Wang & Kelly, 1983; Gordon & Carstens, 1984; Guarino & Summers, 1986; Friesen & Miller, 1986). ICSPs synthesized during each of the four phases have been studied mostly in cultures of *Spodoptera frugiperda* cells infected with *Autographa californica* NPV (AcNPV) or with the closely related *Trichoplusia ni* NPV (TnNPV). Early work identified at least 35 ICSPs synthesized in cells infected with AcNPV and TnNPV (Carstens *et al.*, 1979; Dobos & Cochran, 1980; Wood, 1980; Kelly & Lescott, 1981; Maruniak & Summers, 1981). However, little is known about ICSP synthesis in whole insects. We have used silkworm pupae infected with BmNPV as a stock for preparative purification of some ICSPs (Mikhailov *et al.*, 1986; Voronova *et al.*, 1991). Preliminary experiments suggested that early phase pupae can be used successfully for the expression of foreign genes under the control of the promoter of the very late viral genes whose products are abundant late in infection. The purification of viral products from the pupae, as well as the use of this system in biotechnology, requires additional information about the temporal regulation of protein synthesis and intracellular compartmentalization of ICSPs in infected pupae.

This paper describes a mode of protein synthesis in silkworm pupae following infection with BmNPV. Temporal regulation of ICSP synthesis was demonstrated by SDS–PAGE of polypeptides synthesized in pupae at various times post-infection (p.i.). The subcellular localization of some of the viral products has also been investigated. The very late viral proteins p10 and polyhedrin were shown to be associated with the nuclear...
matrix, suggesting the involvement of nuclear skeletal structures in the biogenesis of BmNPV. The susceptibility of silkworms to BmNPV decreased dramatically during the pupal period to virtually complete resistance in the final pupal stages.

Methods

Insects and viruses. Pupae of the silkworm B. mori were supplied by Dr P. Kulyev (Institute of Zoology, Ashkhabad, Turkmenistan) shortly after larval-pupal ecdysis. BmNPV was prepared from polyhedra and was injected into the pupae as described previously (Onodera et al., 1965; Mikhailov et al., 1986). Infected and uninfected pupae were incubated at 25 °C and sampled daily for biochemical analysis. The pupae were injected with 10 μl of a solution containing 5 or 10 μCi [35S]methionine (400 Ci/mmol) 4 h prior to transfer into liquid nitrogen. Occluded virions were obtained from polyhedra as described by Carstens et al. (1979).

Isolation of nuclei. Nuclei were isolated from silkworm pupae as described previously (Mikhailov et al., 1987) with minor modifications. All procedures were performed at 0 to 4 °C. One or two frozen pupae were homogenized in a Dounce homogenizer in 10 ml 0-3 M-sucrose in buffer A (20 mM-Tris–HCl pH 7-5, 0.2 mM-MgCl2, 2 mM-dithiothreitol, 1 mM-PMSF, 1 mM-leupeptin, 1 μg/ml pepstatin). The homogenate was filtered through nylon and diluted with 10 ml buffer A containing 1:8 M-sucrose. The sample was then layered over 15 ml buffer A containing 1:8 M-sucrose and centrifuged at 50000 g for 1 h. The supernatant was discarded and the nuclear pellet was suspended in 7 to 8 ml buffer A containing 0.3 M-sucrose and centrifuged at 30000 g for 1 h. The supernatant was then layered over 20 ml buffer A containing 1.8 M-sucrose. The nuclear pellet was suspended in 7 to 8 ml buffer A containing 1.8 M-sucrose and centrifuged at 50000 g for 1 h. The supernatant was discarded and the nuclear pellet was suspended in 7 to 8 ml buffer A containing 0.3 M-sucrose. Nuclei were collected by centrifugation at 10000 g for 10 min and finally resuspended in 0.5 ml of the same buffer without MgCl2.

Fractionation of nuclei. One ml of extraction buffer (3 M-NaCl, 10 mM-Tris–HCl pH 7-5, 0.2 mM-MgCl2, 2 mM-dithiothreitol, 1 mM-PMSF, 1 mM-leupeptin, 1 μg/ml pepstatin) was added to 0.5 ml of the nuclear suspension and incubation at 0 °C with stirring was continued for 1 h. The supernatant obtained after centrifugation at 50000 g for 30 min was used as the soluble fraction of nuclei. The concentration of EDTA in the fraction was raised to 2 mM by the addition of neutralized 0.5 M-EDTA and the preparation was dialysed overnight against buffer B (20% glycerol, 10 mM-Tris–HCl pH 7-5, 1 mM-EDTA, 5 mM-2-mercaptoethanol, 1 mM-PMSF, 1 mM-leupeptin, 1 μg/ml pepstatin). The nuclear pellet separated from the soluble fraction of nuclei was treated with 50 μg/ml each of DNase I and bovine pancreas RNase at 20 °C for 1 h. The fraction extracted by nuclease was collected as the supernatant after centrifugation at 30000 g for 30 min. The residual pellet was resuspended in 0.5 ml buffer B and used as the nuclear matrix fraction.

Phosphorylation of proteins. The reaction mixture for protein phosphorylation contained the following ingredients (final concentrations): 20 mM-Tris–HCl pH 8-0, 10 mM-MgCl2, 2 mM-dithiothreitol, 10 μM-[γ-32P]ATP (3 to 5 Ci/mil). Ten μl of the homogenates or subcellular fractions containing 100 μg protein in buffer B was added to the reaction mixture to give a final volume of 10 μl. The reaction was run at 32 °C for 20 min and then stopped by the addition of cold trichloroacetic acid to 10%. Before electrophoresis the pellets, collected by centrifugation at 6000 g for 10 min, were dissolved in denaturation buffer (20% glycerol, 50 mM-Tris–HCl pH 6-8, 2% SDS, 2% 2-mercaptoethanol). Autophosphorylation of proteins of the occluded virions was performed as described by Miller et al. (1983b). Prior to electrophoresis, the preparations of unfractonated nuclei were treated with 50 μg/ml each DNase I and bovine pancreas RNase at 20 °C for 1 h.

Electrophoresis. The samples, each containing 200 or 250 μg protein in denaturation buffer, were denatured and subjected to SDS-PAGE (10% polyacrylamide) as described by Laemmli (1970). The gels were fixed in acetic acid–ethanol and stained with Coomassie blue. For autoradiography of [35S]methionine-labelled proteins the gels were soaked for 15 min in Amplify (Amersham), then transferred to Whatman 3MM paper and dried in vacuo. The gels were exposed to X-ray film PM-B (Tasma) with an intensifying screen.

Detection of DNA-binding proteins. After SDS-PAGE the proteins were transferred to nitrocellulose (Bio-Rad) by electroblotting in buffer (192 mM-glycine, 20% ethanol, 20 mM-Tris, 0.1% SDS, pH adjusted to 8.3 to 8.5). The filter was then incubated for 1 h at room temperature in binding buffer [10 mM-HEPES pH 8-0, 0.1% gelatin, 10% calf serum]. For detection of DNA-binding activity the filter was further incubated for 1 h at room temperature in binding buffer [10 mM-HEPES pH 8-0, 50 mM-NaCl, 1 mM-EDTA, 0-2% gelatin, 32P-labelled calf thymus DNA (106 c.p.m./ml)]. The labelled DNA was prepared by nick translation using Escherichia coli DNA polymerase I and heated to 100 °C for 10 min before the binding procedure. The filter was washed in three changes of buffer (10 mM-HEPES pH 8-0, 0.1 M-NaCl, 1 mM-EDTA) over a period of 2 h, then dried and exposed to X-ray film.

The protein concentration was determined by the method of Lowry et al. (1951).

Results

Protein synthesis in silkworm pupae infected with BmNPV was measured by incorporation of [35S]methionine into nascent proteins at different stages of the infection cycle. The radioactive amino acid was injected 4 h before the pupae were collected to be frozen in liquid nitrogen. Taking into account the duration of the pupal period (approx. 13 days) and that of the infection cycle (approx. 5 to 6 days), the incubation of pupae with [35S]methionine for 4 h may be considered as pulse-labelling.

In uninfected pupae the rate of [35S]methionine incorporation into nascent proteins increased gradually during the pupal period, rising about threefold by the late pupal stages (Fig. 1a). Infection with BmNPV in the early stages of the pupal period induced a cycle of virus-dependent protein synthesis in the pupae. The rate of label incorporation into proteins increased markedly starting 2 to 3 days p.i. Synthesis reached a maximum at 4 to 5 days p.i., dropped on the 6th day and ceased completely on the 7th day p.i. At the peak of synthesis the specific radioactivity of nuclear proteins was about one order of magnitude higher than that of total protein in uninfected cells, indicating that a large portion of the virus-induced proteins accumulated preferentially in the nuclei (Fig. 1b).

The pattern of nascent proteins in BmNPV-infected pupae contrasted markedly with that in uninfected pupae. In virus-infected pupae the rate of synthesis of the host cell proteins was lower than that in uninfected pupae, but incorporation of radioactivity in ICSPs was observed starting from the 3rd day p.i. The major portion
of \(^{35}\text{S}\) methionine incorporated at 4 to 5 days p.i. was associated with a 29K polypeptide (p29) which had an electrophoretic mobility identical to that of polyhedrin, the major component of the virus occlusion bodies (Fig. 5a). This polypeptide constituted \(10\%\) or more of the total pupal protein in the late phase of viral infection. The labelling pattern of ICSPs in female pupae was the same as in male pupae. However, specific radioactivity of ICSPs in females was about one order of magnitude lower than in males, suggesting a higher endogenous pool of methionine or rate of its turnover in females than in males. Therefore, only the results of experiments with male pupae are shown.

To determine the subnuclear localization of ICSPs the isolated nuclei were divided into three protein fractions as described in Methods: (i) the fraction extracted by a buffer containing 2 M-NaCl (soluble fraction), (ii) the fraction solubilized from the extracted nuclei after treatment with RNase and DNase I (nuclease fraction) and (iii) the nuclear skeleton unsolubilized by sequential treatment with NaCl and nucleases (matrix). The protein pattern of all three subnuclear fractions from the control (uninfected) pupae remained unchanged throughout 5 to 6 days of the pupal period. After infection with BmNPV, numerous ICSPs appeared in the nuclear fractions. The specific radioactivity of proteins increased during the infection cycle, initially about twofold in the soluble fraction (3 days p.i.), and about sixfold in the matrix (5 days p.i.). Up to 20 ICSPs with \(M_f\)s from 15K to approximately 110K could be visualized in the gels (Fig. 2). The synthesis of various groups of ICSPs reached a maximum at different phases of viral infection. For example, one of the major radioactively labelled proteins of the soluble fraction, a 35K polypeptide, was synthesized at a maximum rate at 3 days p.i. Synthesis of a 10K polypeptide and p29 reached a peak at 4 and 5 days p.i., respectively. The two latter proteins may be tentatively identified as the most abundant very late viral products p10 and polyhedrin, respectively. Both proteins were present predominantly in the matrix and proved to be the major radioactively labelled ICSPs in this fraction. At the late stages of the infection cycle, \(^{35}\text{S}\) methionine was incorporated exclusively into polyhedrin which constituted more than 90\% of the total protein of the insoluble fraction of the nuclei. The incubation with RNase and DNase I resulted in solubilization of several radioactively labelled ICSPs from the nuclei. The major labelled protein in this fraction was a 31K polypeptide (data not shown).

Two other groups of easily identified ICSPs were virus-specific phosphoproteins and structural proteins of the virus. To identify phosphoproteins, the pupal homogenates and some subcellular fractions (nuclei and nuclear extracts) were incubated in the reaction mixture in the presence of \([\gamma-^{32}\text{P}]\) ATP and cofactors of the kinase reaction (see Methods). Two major acceptors of the label appeared in the pupae after infection. They were polypeptides with apparent \(M_f\)s of 35K (pp35) and 11K to 16K (ppB) (Fig. 3). These polypeptides were efficiently phosphorylated not only in the pupal homogenates, but also in whole nuclei and in the nuclear extracts. Polypeptides pp35 and ppB did not produce abundant protein bands after Coomassie blue staining of the gels. Therefore, their predominant phosphorylation in the preparations from infected pupae indicated that the proteins were very effective substrates in the kinase reaction. The incorporation of label from \([\gamma-^{32}\text{P}]\) ATP into pp35 and ppB was not stimulated by the addition of cAMP (0.1 mM) or Ca\(^{2+}\) (1 mM) to the reaction mixture, and it was inhibited by 40 to 80\% when Mg\(^{2+}\) was replaced by Ca\(^{2+}\). Two polypeptides with electrophoretic mobilities identical to pp35 and ppB were the major acceptors of radioactive phosphate from \([\gamma-^{32}\text{P}]\) ATP during autophosphorylation of the virions purified from infected pupae (Fig. 4, lane 3). These data suggest that phosphoproteins pp35 and ppB are the structural components of the virus. The latter phosphoprotein corresponded to the structural protein bound to radioactively labelled DNA in the DNA-binding assay of the
Fig. 2. Subnuclear localization of proteins synthesized in silkworm pupae after infection with BmNPV. The pupae were infected on the 3rd day of the pupal period and injected with 5 μCi [35S]methionine 4 h before collection of samples at various times p.i. Polypeptide patterns after electrophoresis in 10% SDS–polyacrylamide gels of the soluble fraction of nuclei (a) and the nuclear matrix (b) at various times p.i. are shown (200 μg protein was loaded on each lane). Lanes, 0 to 5, Coomassie blue-stained protein preparations obtained at time of infection (0), and 1 day (1), 2 days (2), 3 days (3), 4 days (4) and 5 days (5) p.i. Lanes 0' to 5', autoradiographs of lanes 0 to 5, respectively. The positions of Mr markers are indicated on the left: ferritin (220K), bovine serum albumin (67K), catalase (60K) and lactate dehydrogenase (36K).
Fig. 3. Identification of phosphoproteins synthesized in silkworm pupae after infection with BmNPV. The pupae were infected on the 3rd day of the pupal period. Phosphoproteins were labelled \textit{in vitro} by incubation of the homogenates (a) and the soluble fraction of nuclei (b) in reaction mixture containing \([\gamma^{-32}P]ATP\) (see Methods) and subjected to electrophoresis on 10\% SDS-polyacrylamide gels (100 \(\mu\)g protein was loaded on each lane). The homogenates and the nuclear extracts were prepared from pupae collected 1 day (1), 2 days (2), 3 days (3), 4 days (4) and 5 days (5) p.i., and uninfected pupae on the 6th (6) and 8th (7) days of the pupal period. Autoradiographs of the gels are shown. The positions of \(M\), markers are indicated on the left: phosphorylase \(b\) (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), trypsin inhibitor (20.1K), \(\alpha\)-lactalbumin (14.4K).

Two experiments were performed to find the stage of the pupal period optimal for production of viral proteins. For these purposes, silkworms were infected with BmNPV at various pupal stages. In the first experiment groups of seven pupae were each infected with BmNPV on the 1st, 3rd, 6th and 10th day of the pupal period. In the second experiment groups of 10 pupae were each infected on the 1st, 3rd, 5th, 7th and 10th day of the pupal period. The virus was prepared from polyhedra by the standard procedure just prior to injection into each group. Progression of viral infection in each pupa...
Fig. 5. Changes in susceptibility of silkworms to BmNPV during the pupal period. Groups of seven pupae each (○) and of 10 pupae each (▲) were infected with BmNPV under standard conditions at various times of the pupal period. The pupae were collected late in infection (5 days p.i.). The amount of the very late viral protein, polyhedrin (P), synthesized in each pupa was determined by Coomassie blue staining and autoradiography after electrophoresis of pupal homogenates (200 µg protein) on 10% SDS-polyacrylamide gels. (a) Analysis of polyhedrin production in five pupae infected on the 1st day (lanes 1 to 5) and in five pupae infected on the 9th day (lanes 6 to 10) of the pupal period. Autoradiographs of the gels are shown. The pupae producing polyhedrin (lanes 1 to 5, 7 and 8) were considered ‘diseased’. (b) The relative number of diseased pupae is shown as the percentage of the total number of pupae infected at each stage of the pupal period.

Discussion

This paper describes protein synthesis in silkworm pupae infected with BmNPV. The main advantage of this model for studying the BmNPV-induced synthesis is the extremely low level of host protein synthesis in the early pupae. In contrast, the viral proteins are synthesized in these pupae very efficiently. Therefore, the infected pupae may be used for the preparative purification of various ICSPs. The data obtained permit identification of only a few ICSPs which were detected by SDS–PAGE. These are the most abundant very late viral products p10 and polyhedrin. p10 associates with fibril-like structures in the nuclei and cytoplasm of infected cells and is presumably involved in cell lysis (Volkman & Zaal, 1990). This protein appears to be associated also with polyhedra (Quant-Russell et al., 1987). Polyhedra produced by mutants lacking the p10 gene have an incomplete polyhedron envelope (Vlak et al., 1988) and are unstable (Williams et al., 1989), suggesting an important role for the protein in morphogenesis of occlusion bodies. In agreement with these data, p10 associates preferentially with skeletal structures of nuclei from the BmNPV-infected pupae (Fig. 2). Polyhedrin appeared also in the nuclear matrix fraction. Polyhedrin was shown by electron microscopy, to accumulate in the nuclei in bodies specific for the occlusion form of the virus (Zemskov et al., 1991). An essential role of association with the matrix has been suggested for the replication of various DNA viruses including baculoviruses (Wilson & Price, 1988). However, this important point should be proved unequivocally. The phosphoprotein ppB was identified as the basic DNA-binding protein of BmNPV according to its electrophoretic mobility and DNA-binding activity. The protein is believed to bind to viral DNA and play a

injected with BmNPV was monitored by visualization of the polyhedrin band by Coomassie blue staining and by autoradiography after SDS–PAGE (Fig. 5a). The extracts for analysis were prepared late in infection (usually on the 5th day p.i.). Silkworms that produced polyhedrin were considered ‘diseased’. The relative number of diseased silkworms following injection of BmNPV at various pupal stages is shown in Fig. 5b. In both experiments the longer the period after larval–pupal ecdisis before the pupae were inoculated, the lower the observed proportion of pupae acquiring polyhedrosis. Sensitivity to the virus decreased dramatically during the pupal period. Most pupae infected late in the period completed metamorphosis, and emerging moths were indistinguishable from the control. The decrease in the frequency of acquired polyhedrosis during the pupal period was accompanied by decreased polyhedrin synthesis in diseased silkworms. This observation was confirmed by densitometric measurement of polyhedrin production on autoradiographs of the gels (Fig. 5a), as well as on the Coomassie blue-stained gels.
crucial role in its packing (Wilson & Miller, 1986; Tweeten et al., 1980). The basic protein of BmNPV comprises 64 amino acids with an M, of about 8K (Maeda et al., 1991). However, an extremely high content of arginine in the protein resulted in its abnormal electrophoretic mobility in the range of 11K to 16K. The phosphoprotein pp35 of BmNPV corresponds presumably to the phosphoproteins pp32 of Orgyia pseudotsugata NPV (Gombart et al., 1989) and pp34 of AcNPV (Whitt & Mannning, 1988), which were found to be associated with the polyhedral envelope of the viruses. However, more evidence is required for the reliable identification of pp35.

This study has demonstrated that the sensitivity of silkworms to BmNPV gradually decreases over the course of the pupal period. In early experiments, Kobayashi et al. (1969) found changes in susceptibility to NPV within silkworm instars. Susceptibility to peroral infection was greatest immediately before and immediately after a moult, but decreased in between. The infection cycle in larvae following ingestion of the polyhedron-derived virions into the columnar cells of the midgut epithelium, which are highly susceptible to the occluded virions. Histolysis of the midgut epithelium proceeds early in the pupal period (Waku & Sumimoto, 1971). Lysis should decrease the number of epithelial cells susceptible to the occluded virions in pupae. The histolysis of other tissues during pupal development may further reduce the number of cellular receptors for the virus, decreasing the efficiency of viral infection. However, a deficit of target cells or cellular receptors for the virus may not be the only reason for the resistance of older pupae. Numerous data suggest that the rate of cellular metabolism may be important in influencing resistance of insect cells to NPV (for review, see Briese, 1986). Whatever the reason for the decrease in susceptibility of silkworms to BmNPV during the pupal period, the earliest pupal stages are the most suitable for the production of viral proteins and probably for the expression of foreign genes under the control of late viral promoters.

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References


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