Changes in DNA Polymerase Activities in Pupae of the Silkworm *Bombyx mori* after Infection with Nuclear Polyhedrosis Virus

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SUMMARY

Changes in DNA polymerase activities were studied in pupae of the silkworm *Bombyx mori* upon infection with nuclear polyhedrosis virus (BmNPV). Two effects were observed in male and female pupae after inoculation of BmNPV: a marked increase in cellular α-polymerase activity and the induction of a new DNA polymerase (BmNPV-polymerase). Activities of both DNA polymerases, α and BmNPV, in the infected pupae increased in parallel, reached a maximum at 72 h post-inoculation and decreased subsequently. The virus infection did not affect the activity of γ-polymerase in the pupae. An increased activity of β-polymerase in the pupae was detected at the late stage of the infection cycle prior to pupal death. The possible roles of the DNA polymerases in BmNPV replication are discussed.

Nuclear polyhedrosis viruses (NPV) of insect species contain a double-stranded, covalently closed circular DNA of approximately 120 to 130 kilobase pairs (Bud & Kelly, 1977; Miller & Dawes, 1979). The mechanism of NPV DNA replication in infected cells is unknown as yet, and information about enzymes that are involved in NPV replication is limited (for review, see Kelly, 1982). It was observed that inoculation of NPV causes a considerable increase in overall DNA polymerase activity in silkworm pupae (Onodera et al., 1968). Stimulation of DNA polymerase by baculoviruses has been demonstrated also in *Spodoptera frugiperda* cells (Kelly, 1981). Whether cellular or virus-specific DNA polymerases were activated during virus infection remains unclear, but the presence of virus-encoded DNA polymerase in cells infected with NPV has been suggested recently (Miller et al., 1981; Wang & Kelly, 1983).

In the present paper, we describe analysis of the activities of different DNA polymerases in pupae of the silkworm *Bombyx mori* after infection with nuclear polyhedrosis virus (BmNPV). It was shown that infection of silkworm pupae by this virus results in the induction of a virus-specific DNA polymerase (BmNPV-polymerase) as well as in a marked increase in the activity of host cell α-polymerase. Purification of BmNPV polymerase from the infected pupae and properties of this enzyme will be described elsewhere (V. S. Mikhailov et al., unpublished).

Preparation of BmNPV and infection of the silkworm pupae were performed as described earlier (Onodera et al., 1965). BmNPV was injected into the pupae on the third day of the pupal period. Control uninfected pupae were injected with the same buffer as the infected pupae but without BmNPV. Infected and uninfected pupae were incubated at 25 °C, sampled daily for biochemical analysis and stored in liquid nitrogen. The mortality of infected pupae was observed 6 days post-inoculation of BmNPV. Extracts of the silkworm pupae for ultracentrifugation in glycerol gradients were prepared as described previously for loach cells (Mikhailov & Gulyamov, 1983). Each extract was prepared from five pupae. An extract from silkworm embryos stored in diapause was obtained by the same method. Aliquots of the extracts (1.5 to 3 mg protein) in 100 μl were layered over 5 ml linear glycerol gradients (10 to 30%). The glycerol gradients were prepared in buffer A for the assay of α-, γ- and BmNPV-polymerases.
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(200 mM-potassium phosphate pH 7.5, 1 mM-EDTA, 5 mM-2-mercaptoethanol), or in buffer B for the assay of β-polymerase (150 mM-KCl, 100 mM-Tris-HCl pH 8.5, 1 mM-EDTA, 1 mM-2-mercaptoethanol). The tubes were centrifuged at 200000 g (average) for 16 to 20 h at 2 °C in a SW50.1 swinging-bucket rotor.

The reaction mixture for the assay of DNA polymerases α and BmNPV contained the following ingredients (final concentrations): 125 μg/ml calf thymus DNA activated with DNase I, 200 μg/ml bovine serum albumin, 8 mM-MgCl₂, 25 μM each of dATP, dCTP, dGTP, 5 μM (20 μCi/ml) [³H]dTTP. In some experiments the activated DNA was replaced by single-stranded phage M13 DNA (10 μg/ml). The reaction mixture for the assay of β- and γ-polymerases contained the following ingredients: 2 units/ml poly(A), 0.1 unit/ml oligo(dT), 200 μg/ml bovine serum albumin, 1 mM-MnCl₂, 5 μM (20 μCi/ml) [³H]dTTP. N-Ethylmaleimide (10 mM) was added to the reaction mixture for the assay of β-polymerase. Another reaction mixture for β-polymerase assay contained all the ingredients as for the α-polymerase assay plus 5mM-N-ethylmaleimide. Twenty-five μl portions of the gradient fractions were added to the reaction mixture to a final volume of 50 μl (assay of β- and γ-polymerases) or 100 μl (assay of α- and BmNPV-polymerases). The reactions were incubated at 37 °C for 1 h and then processed for determination of acid-insoluble radioactivity as described previously (Mikhailov & Gulyamov, 1983). One unit DNA polymerase activity is defined as the amount catalysing the incorporation of 1 nmol dNMP into the acid-insoluble product. Circular single-stranded DNA of phage M13mp8 was prepared as described by Zinder & Boeke (1982).

It has been suggested that BmNPV persists in silkworm pupae in a latent form which may be activated by stress conditions, for example by a heat shock. However, nobody has reported expression of viral genes in silkworm embryo. Therefore, embryo extracts were analysed for identification of silkworm host cell DNA polymerases. Three DNA polymerase activities were detected after ultracentrifugation of the silkworm embryo extract in glycerol gradients (Fig. 1). The largest enzyme has a sedimentation coefficient of 9-6S. This polymerase efficiently uses activated DNA as a primer-template and is inactive on poly(A).oligo(dT). Its activity was reduced by 95% in the presence of 3 mM-N-ethylmaleimide (data not shown). The polymerase was partially purified from silkworm embryos and identified as α-polymerase. The association of DNA primase activity with the silkworm α-polymerase has been demonstrated (Mikhailov et al., 1984). Two minor DNA polymerase activities of the embryo extract were revealed using poly(A).oligo(dT). The first of these (Fig. 1 a) had a sedimentation coefficient of 7-8S, was active in the presence of 100 mM-potassium phosphate and sensitive to N-ethylmaleimide. According to these properties this enzyme was identified as γ-polymerase. The second DNA polymerase (sedimentation coefficient 5-5S) was active at pH 8.5 and N-ethylmaleimide-insensitive (Fig. 1 b). It may be defined as β-polymerase. No DNA polymerase activity on circular single-stranded phage M13 DNA was detected in embryo extracts in the absence of rNTP (Fig. 1 a).

Glycerol gradient ultracentrifugation was used to study changes in DNA polymerase activities in silkworm pupae after infection with NPV. Two effects were observed after inoculation of BmNPV: a marked increase in α-polymerase activity and the induction of a new enzyme (BmNPV-polymerase), which was not detected in uninfected pupae (Fig. 2). The sedimentation coefficient of BmNPV-polymerase was 6-3S. In contrast to α-polymerase, the BmNPV-polymerase uses the single-stranded phage M13 DNA as a primer-template as well as activated DNA. The ability to use phage M13 DNA as a template was due to a nuclease activity co-purified with BmNPV-polymerase (Ataeva et al., 1985).

The activity of BmNPV-polymerase shows a non-linear dependence on the amount of material taken from the gradient fractions in the reaction with activated DNA or phage M13 DNA (Fig. 3). Nevertheless, both templates are suitable for evaluation of BmNPV-polymerase activity. Material from uninfected pupae, which was inactive in the BmNPV-polymerase assay, did not inhibit the enzyme activity in the material from the infected pupae (data not shown). This result suggests the absence of any inhibitors of BmNPV-polymerase in extracts from silkworm pupae.

The time course of DNA polymerase activities in silkworm pupae following infection with BmNPV is shown in Fig. 4. Similar changes were observed in infected male and female pupae.
Fig. 1. Glycerol gradient ultracentrifugation of silkworm embryo extract. The gradients were prepared in buffer A (a) or in buffer B (b). DNA polymerase activities were determined with activated DNA (○, α- and BmNPV-polymerase assay), M13 DNA (■, BmNPV-polymerase assay) and poly(A). oligo(dT) (△, γ-polymerase assay). DNA polymerase β was assayed with poly(A). oligo(dT) in the absence of N-ethylmaleimide (△) or in the presence of 10 mM-N-ethylmaleimide (▴). The left ordinate refers to ○ and □; the right ordinate to ■, △ and ▴.

Fig. 2. Glycerol gradient ultracentrifugation of extracts from silkworm pupae infected with BmNPV. The extracts were prepared from pupae 0 (1), 24 (2), 48 (3), 72 (4) and 96 (5) h after inoculation of BmNPV. DNA polymerase activities were determined with (a) activated DNA (α- and BmNPV-polymerase assay) and (b) M13 DNA (BmNPV-polymerase assay).

The activities of α- and BmNPV-polymerases increased gradually, reaching a maximum 72 h after inoculation, and both activities decreased at the advanced stage of infection. Unlike α-polymerase activity, the activity of γ-polymerase did not change markedly after virus infection. When β-polymerase activity was also measured in infected male pupae, high activity was observed only at the terminal stage of the infection cycle.

Activities of BmNPV-, α- and γ-polymerases were determined in extracts of control pupae. BmNPV-polymerase was not detected in uninfected samples in either polymerase assays with activated DNA or with phage M13 DNA. Activity of α-polymerase in control pupae did not change markedly during the pupal period. Enzyme profiles in glycerol gradients similar to that in line 1 of Fig. 2(a) were obtained in the α-polymerase assay at all stages studied. The specific activity of α-polymerase was 53 units/g protein on the 3rd day of the pupal period (the time of injection), but on the 7th day was 69 units/g protein. These data confirm earlier observations of Onodera et al. (1968), that the overall DNA polymerase activity (predominantly α-polymerase) does not change during the pupal period of the silkworm. In contrast, α-polymerase activity in extracts of infected pupae was 400 to 500 units/g protein on the 6th day of the pupal period (Fig. 4). Therefore, the marked increase in the α-polymerase activity in BmNPV-infected pupae may
Fig. 3. Dependence of BmNPV-polymerase activity on the amount of material added to the reaction mix. Glycerol gradient fractions corresponding to BmNPV-polymerase were pooled after ultracentrifugation, samples from the mixture were added to the BmNPV-polymerase assay with activated DNA (O) or with M13 DNA (●) to a final volume of 100 µl. The extract for ultracentrifugation was prepared from silkworm pupae 72 h after inoculation of BmNPV.

Fig. 4. Changes in DNA polymerase activities in silkworm pupae after inoculation of BmNPV. Activities of DNA polymerases α (△), β (□), γ (■) and BmNPV were determined in (a) female and (b) male pupae. BmNPV-polymerase was assayed with activated DNA (O) and with M13 (●).

be considered to be a result of virus infection. In control pupae the γ-polymerase activity increased slightly during the pupal period in a manner similar to that shown in Fig. 4 for BmNPV-infected species. The specific γ-polymerase activity was 3.7 units/g protein on the 3rd day and 5.3 units/g on the 7th day of the pupal period. Hence, BmNPV infection does not affect γ-polymerase activity in silkworm pupae.

In this paper we have not presented direct evidence that BmNPV-polymerase is actually virally encoded. However, a host cell origin for this enzyme seems improbable. BmNPV-polymerase differs from the silkworm DNA polymerases α, β and γ in sedimentation pattern and enzymic properties. BmNPV-polymerase activity was not detected in the silkworm embryo (Fig. 1). The absence of the enzyme in uninfected pupae also suggests a virus specificity of the BmNPV-polymerase. The data obtained are in agreement with recent observations of a new DNA polymerase in insect cells infected with NPV (Miller et al., 1981; Wang & Kelly, 1983). The parallel increase in BmNPV- and α-polymerase activities in infected pupae (Fig. 4) suggests that both DNA polymerases may be involved in viral DNA replication. On the other hand, the increase in α-polymerase activity may simply be a result of a physiological reaction of pupal cells to virus infection. The unchanged activity of γ-polymerase rules out the possibility that it has an essential role in BmNPV replication. The marked increase in β-polymerase activity at terminal stages of infection was presumably a result of destructive processes in the moribund pupae and
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activation of DNA repair synthesis in the pupal cells. Isolation of BmNPV replication complexes will be important for elucidation of the roles of cellular and virus-specific DNA polymerases in viral DNA replication.

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REFERENCES


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