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Viral Transcription During *Autographa californica* Nuclear Polyhedrosis Virus Infection: a Novel RNA Polymerase Induced in Infected *Spodoptera frugiperda* Cells

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Autographa californica nuclear polyhedrosis virus-specific RNA synthesis in isolated nuclei of *Spodoptera frugiperda* cells in culture was monitored at different times postinfection. Up to 8 h postinfection viral RNA synthesis remained sensitive to 5 μg of α-amanitin per ml. During the course of infection this sensitivity decreased, and at 24 h postinfection RNA synthesis was completely resistant to α-amanitin. DEAE-Sephadex profiles of RNA polymerase isolated at 24 h postinfection showed a new, chromatographically distinct, α-amanitin-resistant form whose kinetics and response to divalent cations differed from those of the host RNA polymerases. The possibility that this enzyme may be responsible for viral late transcription is discussed.

Autographa californica nuclear polyhedrosis virus (AcNPV) is a member of the baculovirus group of insect pathogens. It contains a large, circular double-stranded DNA genome of ca. 8.0 $\times 10^7$ daltons (5, 8) that replicates in the nuclei of infected host cells; the infection leads to the formation of polyhedral inclusion bodies. Physical maps of the genomes of a number of AcNPV strains have been constructed by using restriction endonucleases (5, 11). These maps form the basis for studies of the genetic organization of the NPVs. In particular, it has been shown that late mRNA hybridizes to virtually every region of the viral genome (12). In addition, the locations of several late genes have been established (9). The two major late genes (polyhedrin and a 10-kilodalton protein) have been mapped to *Hin*dIII-F,V and *Hin*dIII-P,Q, respectively (1, 10). However, almost nothing is known about the RNA polymerase(s) that transcribes these viral genes.

In this report we demonstrate that early viral RNA is synthesized by host RNA polymerase II, whereas late viral transcription is carried out by an α-amanitin-resistant enzyme. Furthermore, the transition to late viral transcription is associated with the induction of a novel α-amanitin-resistant RNA polymerase. A comparison of some of the properties of this enzyme with those of the host RNA polymerases is shown.

MATERIALS AND METHODS

Biochemicals. Calf thymus DNA (type I), salmon sperm DNA (type III), penicillin G, streptomycin sulfate, phenylmethylsulfonyl fluoride, DL-dithiothreitol, 2-mercaptoethanol, DEAE-cellulose, and DEAE-Sephadex A-25-120 were purchased from Sigma Chemical Co., St. Louis, Mo. Unlabeled nucleoside triphosphates were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Formamide (reagent grade), dimethyl sulfoxide, and phenol (liquified) were purchased from Fisher Scientific Co., Fairlawn, N.J. The phenol was distilled before use and stored in dark bottles at −20°C. Phosphocellulose P-11 and DE81 filters were obtained from Whatman Inc., Clifton, N.J. Ultrapure ammonium sulfate and the tetrasodium salt of UTP (5-^{3}H; 10 to 25 Ci/mmol) were purchased from Schwarz/Mann, Spring Valley, N.Y. *meta*-Aminobenzyloxymethyl cellulose was purchased from Miles Laboratories, Elkhart, Ind. α-Amanitin was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Liquid scintillation cocktail (3a70) was from Research Products International Corp., Mount Prospect, Ill.

Growth and infection of *Spodoptera frugiperda* cells. Cells were grown and infected as described previously (3) except that 4% fetal calf serum plus 4% calf serum were used in the growth medium. The infection time was measured from the time at which nonoccluded virions were added to the cells.

Isolation of AcNPV DNA. AcNPV DNA was obtained from purified occluded virions from infected *Trichoplusia ni* larvae as described by Miller and Dawes (5).

Isolation of *S. frugiperda* cell nuclei. Cells were collected by centrifugation. They were suspended in 10 ml of hypotonic buffer (10 mM Tris-hydrochloride [pH 7.9], 10 mM NaCl, 1.5 mM magnesium acetate) for 15 min on ice. Triton X-100 was added to make a 0.5% solution. Cells were homogenized with a Dounce homogenizer (Wheaton Scientific, Millville, N.J.) with 10 strokes and centrifuged for 5 min at 1,000 × *g*. The pellet was suspended in 6 ml of hypotonic buffer. A 6-ml volume of sucrose buffer (1 M sucrose, 0.025 M Tris [pH 7.9], 1 mM $CaCl_2$) was underlaid by slowly

pipetting it down the wall of the tilted tube. The nuclei were then sedimented by centrifuging for 30 min at 1,000 × *g*. The pellet containing the nuclei was suspended in 0.3 ml of TGED buffer (50 mM Tris-hydrochloride [pH 7.9], 35% glycerol, 0.1 mM EDTA, 0.5 mM dithiothreitol). The nuceli were clean as judged by microscopic examination.

RNA synthesis in isolated nuclei. Reactions were carried out as described by Grula et al. (3) except that 2×10^7 nuclei per reaction were used. The reactions were incubated for 10 min at 37°C and then stopped by adding 50 μl of 0.5 M sodium acetate, 50 μl of 5% sodium dodecyl sulfate, and 70 μl of TE buffer (0.1 M Tris [pH 7.9], 1 mM EDTA).

The newly synthesized RNA was extracted as described by Landes (G. M. Landes, Ph.D. dissertation, University of Kansas, Lawrence, 1978) by adding an equal volume (500 μl) of H_2O-saturated phenol at 80°C to the mixture. This was incubated for 10 min at 80°C and vortexed twice during this period. The reactions were centrifuged for 5 min in an Eppendorf microcentrifuge in the cold. The aqueous phase was transferred to another microcentrifuge tube and extracted once with 1 volume of $CHCl_3$. The RNA was precipitated from the aqueous phase by adding 2.2 volumes of cold ethanol, 2 μg of yeast RNA, and 0.1 volume of 3 M sodium acetate.

Before RNA-DNA hybridization, the labeled RNA was separated from the unincorporated [^{3}H]UTP by using a small G-75 Sephadex column equilibrated with TE buffer. The [^{3}H]RNA was suspended in 100 μl of TE buffer and poured into the column. Fractions of 100 μl were collected and eluted with 100 μl of TE buffer each, and portions of each fraction were counted in 3a70 cocktail in a scintillation counter. The first peak contained the [^{3}H]RNA, and the corresponding fractions were combined. To precipitate the [^{3}H]RNA, 2 volumes of ethanol and 0.1 volume of sodium acetate were added.

Coupling NPV-specific DNA to cellulose. DNA was coupled to *meta*-aminobenzyloxymethyl cellulose by the procedure described by Noyes and Stark (7).

Hybridization of RNA to NPV DNA. The precipitate of [^{3}H]RNA was suspended in 300 μl of hybridization buffer (50% formamide, 0.6 M NaCl, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 0.1 M Tris [pH 7.9]), and the radioactivity of a small sample was measured to determine the input for the hybridization. Each RNA sample was hybridized in duplicate to NPV DNA-cellulose and to a control of calf thymus DNA-cellulose. All hybridizations were performed in a total volume of 0.2 ml in sterile polyethylene microcentrifuge tubes. DNA-cellulose was suspended in 50% formamide buffer containing 0.5 mg of yeast RNA and 0.2 mg of polyadenylic acid per ml. RNA was added, and the suspension was mixed thoroughly, heated to 80°C for 1 to 2 min, and incubated at 37°C for 18 h on a rocker platform (Bellco Glass, Inc., Vineland, N.J.).

After hybridization, the samples were placed on ice and centrifuged for 1 min in a microcentrifuge. The cellulose was washed twice by suspension in 0.2 ml of formamide buffer and twice with ice-cold 2× SSC (1× SCC is 0.15 M NaCl plus 0.015 M sodium citrate). The hybridized RNA was eluted two times with 100 μl of 99% formamide–0.1% sodium dodecyl sulfate, followed by heating for 1 min at 60°C. The combined eluates were ethanol precipitated and stored at −20°C. The precipitate was centrifuged, suspended in 50 μl of TE buffer, and counted to determine the amount of [^{3}H]RNA that hybridized to the DNA-cellulose.

RNA polymerase assays. RNA polymerase reactions were done as described by Grula et al. (3)

Preparation of ion-exchange resins. DEAE-cellulose and P-11 phosphocellulose were prepared as described by Green et al. (2) by washing first with acid and then with base and removing fines by aspiration.

DEAE-Sephadex was prepared by washing with 50 mM Tris-hydrochloride, pH 7.9, and equilibrating with TGED buffer before it was used.

RNA polymerase purification. All steps were carried out at 4°C. Cells were harvested and suspended in 2 ml of TGEDP buffer (TGED buffer plus 1 mM phenylmethylsulfonyl fluoride) containing 0.3 M ammonium sulfate. They were sonicated with a Bronwill Biosonic IV (VWR Scientific, Inc., San Francisco, Calif.) for 2 min, with 15-s pulses on ice. The extract was diluted to 6 ml with the same buffer and centrifuged for 45 min at 90,000 × *g* (6). A freshly prepared 10% solution of streptomycin sulfate was added dropwise to the supernatant to a final concentration of 1.25%, with continuous stirring for 30 min on ice. The mixture was centrifuged for 90 min at 118,000 × *g*. The resulting supernatant was dialyzed for at least 4 h against TGEDP without salt. The precipitate was pelleted by centrifuging for 20 min at 90,000 × *g* in a Beckman L5-50 ultracentrifuge. The clear supernatant was loaded into a phosphocellulose column previously equilibrated with TGEDP. RNA polymerases were eluted with a linear 25 to 450 mM ammonium sulfate gradient in TGEDP. Fractions containing the polymerase activity were pooled and diluted to a concentration of 35 mM or less and poured into a DEAE-cellulose column equilibrated with TGEDP. RNA polymerases were eluted in a single peak with a linear 25 to 450 mM ammonium sulfate gradient in TGEDP. The appropriate fractions were pooled and either dialyzed or diluted to 35 mM ammonium sulfate. The pool was applied to a DEAE-Sephadex column, and a linear gradient was performed as described above. The separated forms of RNA polymerase eluted from DEAE-Sephadex were stored at −80°C. Salt concentrations of eluted fractions were measured in a Radiometer conductivity meter (The London Co., Cleveland, Ohio).

RESULTS

Viral RNA synthesized in isolated nuclei. RNA was synthesized in the presence and absence of 3 μg of α-amanitin per ml in nuclei isolated 6, 7, 8, 9, 12, 16, 18, 20, and 24 h postinfection (p.i.). RNA was extracted and hybridized to virus-specific DNA coupled to *meta*-aminobenzyloxymethyl cellulose (10 μg of DNA per mg of cellulose). The results of this experiment are shown in Fig. 1. The efficiency of hybridization was 50% and was determined from the percentage of counts hybridized by using a range of known inputs of viral [^{3}H]RNA. This efficiency value was used to compute the percentage of viral RNA in each sample. In all cases, the viral RNA amounts were limiting, as shown by control experiments with various RNA inputs (data not shown).

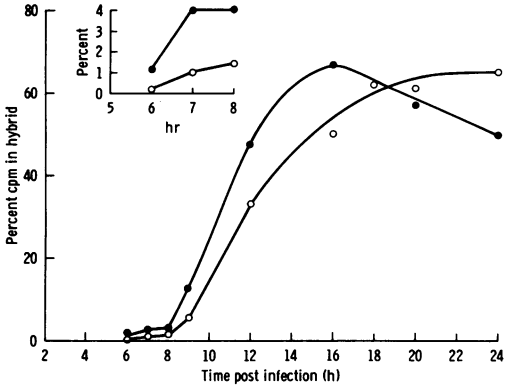

FIG. 1. Synthesis of virus-specific RNA in isolated, infected *S. frugiperda* cell nuclei. Nuclei were isolated from infected cells at various times p.i. and pulse-labeled with [^{3}H]UTP for 10 min in the presence and absence of 5 μg of α-amanitin per ml. The labeled RNA was extracted, hybridized to viral DNA-cellulose, and then eluted as described in the text. The percentage of labeled RNA that hybridized to the viral DNA was plotted as a function of time p.i. Data are corrected for nonspecific hybridization of the [^{3}H]RNA samples to calf thymus DNA. RNA synthesized in infected nuclei in the absence of α-amanitin (●) and in the presence of 5 μg of α-amanitin per ml (○). Inset, Expansion of 6- to 8-h portion of graph.

Before 6 h p.i. no significant amounts of viral [^{3}H]RNA were detected. At 8 h p.i. only 4% of the total RNA produced in nuclei was viral (Fig. 1 inset). This early viral RNA synthesis was sensitive to α-amanitin, and the early viral RNA was therefore probably made by host polymerase II. As infection proceeded, viral RNA synthesis increased rapidly for the next 4 h and α-amanitin sensitivity gradually decreased. After 12 h p.i. the rate of accumulation of viral RNA slowed. It reached a maximum at 16 h p.i. when at least 70% of the total RNA synthesized in nuclei was viral.

Purification of RNA polymerases from normal and virus-infected *S. frugiperda* cells. DEAE-Sephadex profiles of RNA polymerases from infected and uninfected cells are shown in Fig. 2. Fractions were assayed in the presence and absence of 1 μg of α-amanitin per ml and NPV DNA at a concentration of 10 μg/ml.

Uninfected cells (Fig. 2a) showed a typical pattern of RNA polymerases I, II, and III. Forms I and III were resistant to α-amanitin, and form II was sensitive to α-amanitin. However, the infected-cell profiles (Fig. 2b) showed a new form, which was not present in normal, uninfected cells, overlapping RNA polymerase II. This virus-induced form was resistant to α-amanitin concentrations as high as 1 mg/ml (data not shown) and therefore could be distinguished from host RNA polymerase II.

Figure 3 shows the results of an experiment in which a different preparation of RNA polymerase from infected cells was tested with salmon sperm DNA instead of viral DNA. The virus-induced polymerase appeared just as in the experiment with viral DNA. The same behavior was observed with calf thymus DNA (data not shown).

Characterization of the different forms of RNA polymerases. (i) Metal ion requirements. Purified RNA polymerases were analyzed at different concentrations of $MgCl_2$ and $MnCl_2$. Figures 4 and 5 show the effects of Mg^{2+} and Mn^{2+}, respectively. The more pronounced difference was in the response to Mn^{2+}; the host polymerases showed optima at 4 mM (polymerases I and II) and 3 mM (polymerase III), compared with the 5 mM optimum of the virus-induced polymerase.

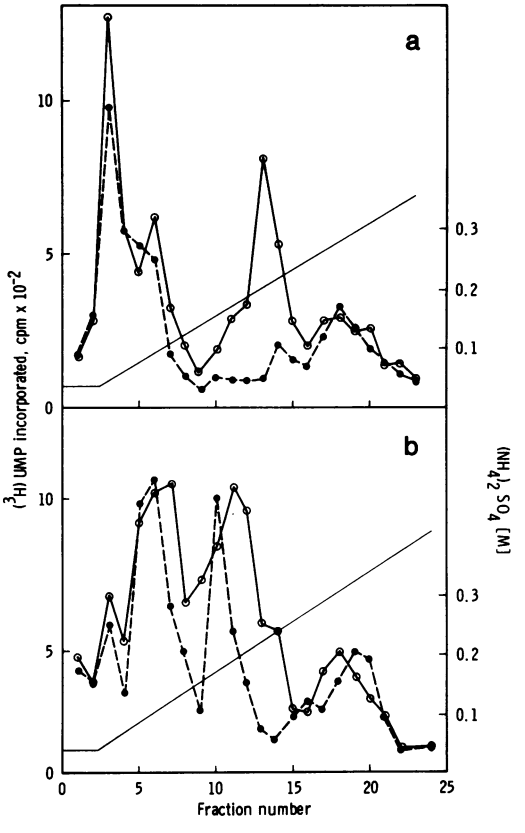

FIG. 2. DEAE-Sephadex elution profiles of RNA polymerases from uninfected cells (a) and cells 24 h p.i. (b). Fractions were assayed in the presence (●) and absence (○) of 1 μg of α-amanitin per ml. RNA polymerases were solubilized as described in the text. Activity was measured by using 10 μg of NPV DNA per ml as the template. The elution gradient is indicated (——).

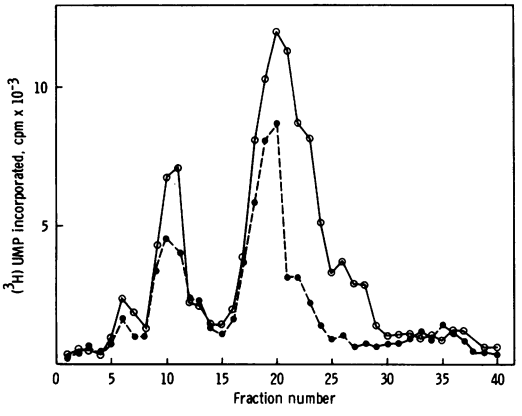

FIG. 3. DEAE-Sephadex elution profile of RNA polymerases from cells 24 h p.i. Reactions were carried out with 10 μg of salmon sperm DNA per ml in the presence (●) and absence (○) of 1 μg of α-amanitin per ml.

(ii) Time course of RNA polymerase activity. Figure 6 shows the time course of activity of each RNA polymerase. Whereas all three host polymerases showed progressive loss of activity over a 1-h period, the virus-induced polymerase remained fully active for at least 1 h.

Host RNA polymerases I, II, and III from infected cells showed the same characteristics as did the corresponding host RNA polymerases from normal cells (data not shown). All characterization experiments carried out with α-amanitin-resistant forms were done in the presence of 1 μg of α-amanitin per ml to eliminate any effect of contaminating RNA polymerase II. These experiments were performed by using 10 μg of calf thymus DNA per ml as the template.

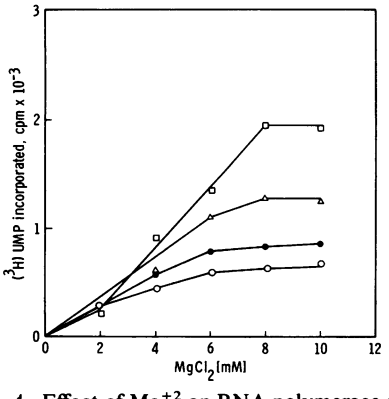

FIG. 4. Effect of Mg^{+2} on RNA polymerase activity. Activity was assayed by using calf thymus DNA as the template at the indicated $MgCl_2$ concentrations. α-Amanitin-resistant RNA polymerases were assayed in the presence of 1 μg of α-amanitin per ml. Symbols: ○, polymerase I; □, polymerase II; ●, polymerase III; △, virus-induced polymerase.

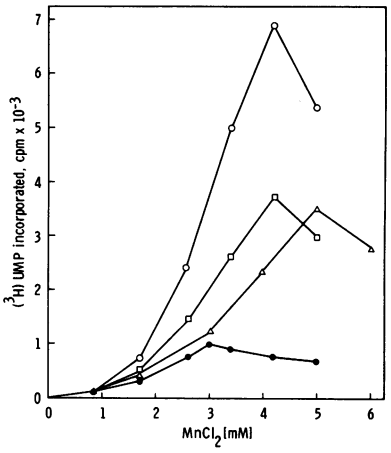

FIG. 5. Effect of Mn^{+2} on RNA polymerase activity. Assays were performed by using calf thymus DNA as the template at the indicated $MnCl_2$ concentrations. α-Amanitin (1 μg/ml) was added to assay RNA polymerases I and III and virus-induced RNA polymerase. RNA polymerase II was assayed as described in the text, with no α-amanitin. Symbols are as described in the legend to Fig. 4.

DISCUSSION

The data reported here establish the existence of an α-amanitin-resistant RNA polymerase induced by NPV infection of *S. frugiperda* cells. The partial purification through DEAE-Sephadex chromatography allows the separation of this novel form from other α-amanitin-resistant forms. Although the novel RNA polymerase cannot be completely separated from RNA polymerase II by this procedure, it becomes evident when α-amanitin is added to the reaction mixture.

In vitro experiments show that the virus-induced polymerase exhibits features that are not characteristic of host RNA polymerases. The activity of the virus-induced enzyme is constant for at least 1 h, whereas host RNA polymerase activities remain linear for 10 min, at most. The novel form, as well as the host RNA polymerases, prefers Mn^{2+} to Mg^{2+}, but it has an Mn^{2+} optimum of 5 mM, which is higher than that required for any of the host RNA polymerases.

The transcription experiments performed in nuclei show that early viral transcription is sensitive to α-amanitin and therefore presumably carried out by host RNA polymerase II, the only α-amanitin-sensitive enzyme. However, late viral transcription is resistant to α-amanitin and therefore performed by an enzyme other than host RNA polymerase II. These results are similar to those reported previously (3) except that they show a difference in α-amanitin sensi-

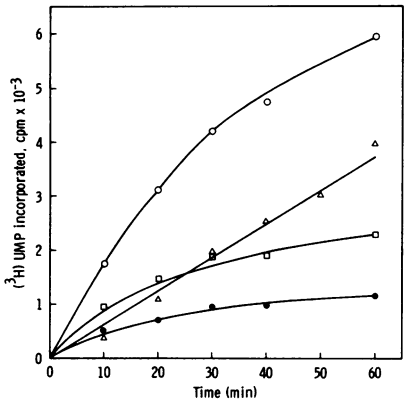

FIG. 6. Time course of RNA polymerase activities. Assays were performed as described in the text by using calf thymus DNA as the template. Symbols are as described in the legend to Fig. 4.

tivity between early and late transcription. The present studies used a more reliable hybridization assay and many early time points to detect this difference.

The apparent stimulation of viral RNA synthesis by α-amanitin late in infection could be at least partially artifactual for two reasons. First, we compared viral RNA synthesized in the presence and absence of α-amanitin as a percentage of total RNA synthesized rather than on an absolute basis. This was necessary to control for loss of RNA during purification before hybridization. However, if there is considerable RNA polymerase II activity, α-amanitin will inhibit it and therefore lower the total RNA synthesis. If α-amanitin has no effect on viral RNA synthesis, the lowering of total RNA synthesis will cause an apparent increase in the proportion of RNA synthesis that is viral. This is probably not a serious problem, because RNA polymerase II is a minor component of the total polymerase activity in late infected nuclei. Second, a source of apparent stimulation would be the release of transcription factors from polymerase II by α-amanitin. These could then be used by the α-amanitin-resistant polymerases. We cannot rule out this possibility.

In a previous study DEAE-Sephadex elution profiles of RNA polymerases from infected and uninfected cells were compared, and no significant differences were found (3). The present studies used a different method of polymerase extraction which apparently retained the virus-induced polymerase activity much better.

The switch from α-amanitin-sensitive to α-amanitin-resistant transcription is gradual, beginning at ca. 7 h p.i. and ending by ca. 18 h p.i. The onset of this switch coincides well with the onset of viral DNA replication at about 6 to 7 h p.i. (9) and therefore with the switch from early to late transcription.

These data are compatible with the following model. Early in infection, the viral genes are transcribed by host RNA polymerase II. One or more early genes code for a virus-specific α-amanitin-resistant RNA polymerase or for factors that modify one of the host polymerases. The virus-induced or virus-modified polymerase then plays a major role in late transcription.

One objection to this interpretation is that the novel polymerase does not appear to be the predominant activity late in infection. This may simply reflect the fact that host RNA is still being actively synthesized late in infection. This is implied by the finding that, even when viral RNA synthesis is at its peak, the contribution of host RNA synthesis in isolated nuclei is still ca. 30%. It is also possible that considerable loss of the virus-induced polymerase occurs during enzyme purification. This notion is supported by studies on the occasional preparation in which separation of polymerase forms occurs at the DEAE-cellulose stage. In these cases, the largest peak of polymerase is an α-amanitin-resistant form that elutes after the RNA polymerase I peak. Upon rechromatography on DEAE-Sephadex, this polymerase peak elutes in the position of the virus-induced enzyme and has the response to Mn^{2+} expected for that enzyme, but it is greatly reduced in activity compared with the other forms (data not shown).

Further characterization of this virus-induced RNA polymerase should shed light on the mechanisms involved in transcription switching in this interesting host-virus system.

ACKNOWLEDGMENTS

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