Induction of Poxvirus Ribonucleic Acid Polymerases

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Two distinct ribonucleic acid polymerase activities were induced in HeLa cells by poxvirus infection. These activities differ both in their properties and the time of their appearance after infection. One catalyzes the dAT (copolymer of deoxyadenylate and deoxethylmidylate)-primed conversion of adenosine triphosphate and uridine triphosphate into an acid-insoluble product. This enzyme is detectable only if deoxyribonucleic acid synthesis has been blocked. In contrast, the accumulation of progeny genomes is a necessary condition for induction of the second enzyme. The latter activity, which is unmasked by detergent treatment, is found exclusively in maturing virus particles. The possibility that both enzymes are involved in transcribing the viral genome is discussed.

A deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase activity is an integral component of the poxvirus (7, 11). Cores (i.e., virus that has been only partially uncoated), as well as whole virus particles, will mediate the in vitro synthesis of virus-specific messenger RNA (mRNA; reference 7). Upon infection of cells, it is presumably this enzyme which transcribes that part of the viral genome coding for "early" viral functions (i.e., those transcribed in the absence of DNA synthesis). The rate and duration of transcription of these functions is determined by factors affecting the uncoating of virus particles. When uncoating is prevented by the use of inhibitors, transcription of early viral mRNA is continuous (8, 13). On the other hand, if uncoating is allowed to proceed in the presence of inhibitors of viral DNA synthesis, the initial rate of mRNA synthesis is rapidly reduced to a low level (13, 14).

Under the latter conditions, even though both input polymerase and naked viral DNA are present, late species of viral mRNA are not transcribed (12). This raises the question of whether "late" mRNA is transcribed by the input enzyme once the DNA replicates, or whether new polymerase molecules must be synthesized for such transcription. We assume that information for the synthesis of the virion polymerase is encoded within the viral genome. One would expect, therefore, to find an enhanced RNA polymerase activity which is due to synthesis of enzyme destined to be packaged into progeny virus. These two problems prompted an investigation of RNA polymerase induction in poxvirus-infected cells.

MATERIALS AND METHODS

Cells, medium, virus, and infection of cells. HeLa S3 cells were grown in suspension in Eagle's medium supplemented with 5% calf serum. Highly purified stocks of vaccinia virus (strain WR) containing 2 $\times$ 10^10 to 3 $\times$ 10^10 elementary bodies (EB)/ml (3) were used throughout. The procedure for infection was essentially that described by Becker and Joklik (1).

Preparation of cytoplasmic extracts. Crude cytoplasmic extracts were prepared from samples of 2 $\times$ 10^10 cells. The cells were collected by centrifugation, washed with Earle's saline, and allowed to swell for 10 min in 1 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0. The cells were disrupted with a Dounce homogenizer, and the cytoplasmic fraction was obtained by centrifuging the homogenate at 500 $\times$ g for 2 to 3 min. The protein concentration of this fraction varied less than 5 to 10% throughout the course of these experiments.

Assay for polymerase activity. Samples (0.1 ml) of crude cytoplasmic extracts were assayed (i) without further treatment, or (ii) after incubation (15 to 30 min at 4 C) with mercaptoethanol (1%) or mercaptoethanol plus detergent (1%). The standard reaction mixture contained (in micromoles): Tris buffer, pH 8.5, 30; MgCl2, 3; mercaptoethanol, 2; adenosine triphosphate (ATP), 1; guanosine triphosphate (GTP), 1; cytosine triphosphate (CTP), 1; and 3H-uridine triphosphate (UTP), 0.1 (specific activity 1.2 $\times$ 10^6 counts per min per amole), in a final volume of 0.25 ml. After incubation for 1 hr at 37 C, the reaction was stopped by the addition of 3 ml of 3.5% perchloric acid containing 0.1 M pyrophosphate. Acid-

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insoluble material was collected on membrane filters (Millipore Corp., Bedford, Mass.), washed with 15 to 20 ml of cold 1 N HCl containing 0.1 M pyrophosphate, and prepared for scintillation spectrometry.

dAT (a copolymer of deoxyadenylate and deoxycytidylymidylate)-primed reaction. Conditions of the reaction were as described above for the polymerase assay, except that GTP and CTP were omitted, and dAT (a gift from M. Chamberlin, who suggested the use of the compound) was present at a final concentration of approximately 0.5 optical density (OD)$_{260}$ units/ml.

Preparation of viral DNA. Primer DNA (WR) was prepared according to the methods of Joklik (4).

RESULTS

Induction of RNA polymerase. An attempt was made to demonstrate an increase in RNA polymerase activity after poxvirus infection. Cells were infected with WR (2,000 EB/cell) and samples were taken at intervals for preparation of crude cytoplasmic extracts. These were used for assay of polymerase activity. No increase in RNA polymerase activity was detected in the absence of detergent (curve D, Fig. 1). However, if these same extracts were made 1% with respect to NP40 (Shell Nonidet), a marked increase between 4 and 24 hr postinfection was detected (curve A, Fig. 1). Similar results were obtained by treating the extract with Brij 58 (Atlas Chemical Co.) or Triton X-100 (Calbiochem) but not with sodium deoxycholate or sodium dodecyl sulfate. This enzyme activity did not require or respond to the addition of primer DNA. The increase in activity was completely inhibited by adding cytosine arabinoside (CAR) at 30 min postinfection (curve D, Fig. 1). The final level of activity in the presence of CAR was dependent upon the time of addition of this inhibitor, as shown in curves B and C, Fig. 1.

The fate of the input polymerase during the first few hours after infection is shown on an expanded scale in Fig. 2. The activity of this enzyme, which drops about 50%, can be detected in extracts which have not been treated with detergent (curve C). The residual activity (at 2 to 3 hr postinfection) is probably due to the presence of virus which has not yet been fully uncoated. In no case could we obtain evidence for the release of the virion polymerase in a form which would respond to the addition of exogenous primers. Furthermore, the activity of these extracts was the same whether viral DNA synthesis had been prevented or not. Clearly, this result does not reflect the pattern of mRNA synthesis in vivo (13).

Effect of inhibiting protein synthesis on induction of polymerase. The effect of inhibiting protein synthesis was investigated by using cycloheximide. Levels of cycloheximide as low as 25 µg/ml inhibited protein synthesis in infected cells by over 95% in 30 min. Cycloheximide (100 µg/ml) was added to infected cells at selected times postinfection. Samples of cells were subsequently

![Fig. 1. Induction of RNA polymerase activity in WR-infected HeLa cells. Polymerase activity of infected cell extracts treated with mercaptoethanol (1% final concentration) but no detergent (●); activity of extracts made to 1% with both NP40 and mercaptoethanol (○); activity of NP40- and mercaptoethanol-treated extracts after addition of CAR to cells at 0.5 hr (★), 2 hr (●), 5 hr (□) postinfection. Activity is expressed as counts per min of 3H-uridine monophosphate incorporated per hr per 0.01 ml of extract.](http://jvi.asm.org/)

![Fig. 2. Decrease in input polymerase activity after infection. Polymerase activity of infected cell extracts treated with both NP40 and mercaptoethanol (○); uninfected cell extracts treated in the same way (□). Polymerase activity of infected cell extracts without mercaptoethanol treatment (▲); with mercaptoethanol but without detergent (●).](http://jvi.asm.org/)
harvested and cytoplasmic extracts were assayed for polymerase activity by use of the detergent NP40. Inhibition of protein synthesis prior to 4 hr postinfection completely prevented an increase in polymerase activity (curve F, Fig. 3). If inhibitor was added later, then activity continued to increase for several hours (curves E, C, and B, Fig. 3). The effect of cycloheximide was completely reversible (curve D, Fig. 3).

Location of induced polymerase activity. The location of the induced polymerase activity was investigated by techniques which have previously established the sedimentation characteristics of virions and cores (5). At 6 and 10 hr postinfection, cytoplasmic extracts were prepared from 6 × 10^7 WR-infected cells; they were applied to sucrose gradients (25 to 40% in 0.01 M Tris, pH 8.0) and centrifuged at 70,000 × g for 50 min. The gradient was fractionated, the OD_{260} was recorded automatically, and the polymerase activity was determined on 100-μl samples of each fraction. The results (Fig. 4) show that all polymerase activity coincides with the position of virions or cores. No polymerase activity (primable or nonprimable) was found in any other fraction.

dAT-primed activity. If cells are assayed at any time after infection (Fig. 1 and 2), the only polymerase activity measured by our assay is (i) that remaining in whole virus or subviral particles that have not completely uncoated, and (ii) that which has been packaged into progeny particles. That is to say, polymerase is not detectable in a soluble form, even in the presence of various DNA primers (see Fig. 5).

It seemed likely that an induced polymerase should exist in a soluble form. Chamberlin and Berg (2) have shown that dAT is a highly efficient primer for Escherichia coli RNA polymerase. Therefore, we employed this primer under the following conditions. (i) Cells were infected in the presence of 5′-fluorodeoxyuridine (FUdR; 10^{-5} M) or CAR (10^{-4} M) to magnify the levels of early-induced enzymes; (6, 9); (ii) GTP and CTP were omitted from the reaction mixture. Under these conditions, an increase in dAT-primed activity was detected. Both ATP and UTP were required for activity which was equally distributed between soluble (supernatant fluid from 100,000 × g centrifugation) and particulate fraction. Neither detergent nor mercaptoethanol stimulated the activity.

Effect of inhibitors on the induction of the dAT-primed activity. Increase in the dAT-primed activity (curve A, Fig. 6) is detectable in cells only if viral DNA synthesis is prevented. Reversal of FUdR inhibition by addition of thymidine (10^{-4} M; curves D and E, Fig. 5) rapidly arrested the increase in enzyme activity. Addition of cycloheximide (25 μg/ml) at any time after infection rapidly inhibited protein synthesis and also terminated the increase in enzyme activity (curves B and C, Fig. 5).

**FIG. 3.** Effect of inhibitors of protein synthesis on induction of RNA polymerase. Postinfection increase in polymerase, no inhibitor added (C); cycloheximide added at 4 hr ( ), 6 hr ( ▲ ), 8 hr ( □ ), 12 hr ( ▣ ), or at 4 hr, followed by removal of inhibitor at 8 hr ( • ) postinfection. In all cases, extracts were treated with NP40 and mercaptoethanol.

**FIG. 4.** Sedimentation of polymerase activity in sucrose gradients. Unbroken lines, optical density at 260 nm recorded automatically. Broken lines, polymerase activity. Cytoplasmic extracts were prepared at 6 and 10 hr postinfection.
activity is in a form that requires detergent plus mercaptoethanol to unmask it. The kinetics of increase in this polymerase are coincident with those of formation of progeny virus and all activity is in a form that sediments in sucrose gradients with the characteristics of progeny virus. It is remarkable that the polymerase activity of purified virions can be unmasked by mercaptoethanol treatment alone (7), whereas the activity of the same enzyme in crude extracts requires the addition of detergent. Detergent also stimulates the activity of purified virus (unpublished results). The detergent effect could be merely due to permeability changes. It might also be due to inactivation of a regulatory compound involved in restricting the activity of progeny particles before their release from the cell. The possibility that adenosine triphosphatase activities are involved is being investigated.

Although we cannot determine the exact time at which the virion enzyme is synthesized, it is clear that the appearance of polymerase activity depends upon DNA synthesis. After about 4 hr postinfection, a pool of this enzyme does indeed exist in a form which we cannot assay directly; however it is clear that detectable activity can increase in the absence of continued protein synthesis (Fig. 3). These findings suggest that the synthesis of some other late protein(s) is required to activate the virion polymerase. We suggest that the increase in activity in the absence of protein synthesis represents the packaging of polymerase into progeny particles, and only in that state is it measurable.

The increase in dAT-primed activity requires continuous protein synthesis (Fig. 6). This induced enzyme appears to be under viral control, since no detectable postinfection increase occurs if viral DNA synthesis takes place (Fig. 6; reference 10). Although a direct role of the dAT-primed activity in transcription has not been demonstrated, the following observations imply that the viral DNA can at least interact with this enzyme. (i) If DNA synthesis takes place, no dAT-primed activity is detectable. (ii) If inhibition of DNA synthesis is reversed after the dAT-primed activity appears, then further increase is arrested and the activity gradually declines. (iii) Addition of exogenous viral DNA to the reaction mixture inhibits the dAT-primed reaction by over 90% (unpublished results).

In spite of these results, it is clear that conditions under which input polymerase, uncoated viral DNA, and dAT-primed enzyme coexist are not sufficient for late mRNA synthesis. Obviously, other factors are involved. We are trying to establish conditions necessary for late-messenger synthesis.

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