JOURNAL OF VIROLOGY, Apr. 1972, p. 621-626
Vol. 9, No. 4

Transcription of the Adenovirus Genome by an α-Amanitine-Sensitive Ribonucleic Acid Polymerase in HeLa Cells

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Received for publication 21 December 1971

The properties of the ribonucleic acid (RNA) polymerase activity which transcribes the major portion of the adenovirus genome were studied. Nuclei were prepared from infected cells and incubated in vitro. Virus-specific RNA was determined by hybridization to adenovirus deoxyribonucleic acid (DNA). Adenovirus DNA is transcribed principally by an activity which resembles closely polymerase II of the host cell. This activity is inhibited by α-amanitine and stimulated by $(NH_4)_2SO_4$. Its product is high-molecular-weight heterogeneous RNA. The polymerase activity measured early in infection (3 to 5 hr) resembles that found late in infection (16 to 18 hr).

The macromolecular metabolism specific to adenovirus growth resembles that of the host cell in many ways (4). Adenovirus deoxyribonucleic acid (DNA) replicates and adeno-specific ribonucleic acid (RNA) is transcribed in large molecules in the nucleus of the infected cell (5). The high-molecular-weight RNA is apparently cleaved, and polyadenylic acid is added. The product is then exported to the cytoplasm where it is translated as messenger RNA by the host cell ribosomes (5, 12–14; M. Hirsch, *unpublished observations*). Adenovirus-specific proteins then reassociate with adenovirus DNA in the nucleus to form the mature virus structures (19, 20). Thus, adenovirus offers a model system in which relatively few genes are expressed through mechanisms which resemble those of the uninfected cell.

In this report, the transcription of the adenovirus genome in the infected cell is examined. Previous investigators have shown the existence of two and possibly three distinct RNA polymerase activities associated with the mammalian cell nucleus (16–18). The polymerase activities II and III, as designated by Roeder and Rutter (18), are located in the nucleoplasm. A previous report suggested that polymerase II appears to be responsible for the major fraction of heterogenous RNA (Hn-RNA) synthesis in the uninfected cell (22). In this report, it is shown that an activity resembling polymerase II accounts for the major fraction of the transcription of the adenovirus genome when nuclei from infected cells are examined in vitro. A similar finding was obtained independently by Wallace and Kates (21).

MATERIALS AND METHODS

Cells and virus. HeLa S-3 cells were grown in suspension culture in Eagle medium supplemented with 7% horse serum. Adenovirus type 2 was obtained from M. Green. Infections were performed by concentrating cells into one-twentieth of the original volume of medium. Virus was then added at a multiplicity of approximately 200 plaque-forming units per cell. The suspension was shaken for 1 hr at room temperature and brought up in fresh medium. The virus was purified and DNA was extracted according to methods previously described (1).

Preparation of RNA. Hn-RNA from infected cells was labeled and extracted as described previously (9). In all experiments ribosomal RNA synthesis was suppressed by treating cells for 30 min before labeling with 0.04 μg of actinomycin D per ml.

For incubation of isolated nuclei in vitro, nuclei were prepared as described previously, except that the lysis buffer ionic composition was changed to 10 mM $MgCl_2$, 24 mM KCl, 10 mM tris(hydroxymethyl)-aminomethane (Tris), *p*H 8.0 (22). The nuclei from infected cells tended to aggregate at the lower ionic strength used for uninfected cells. Incubation was normally carried out for 10 min at 32 C. RNA was analyzed on sodium dodecyl sulfate (SDS) sucrose gradients as described previously (9).

Hybridization. DNA-RNA hybridization was carried out under conditions of DNA excess to insure the detection of all viral sequences. Adenovirus was prepared and DNA was extracted by the method of Doerfler (1). Purified ^{14}C-labeled DNA was attached to nitrocellulose filters (Carl Schleicher and Schuell Co.) as described previously (3). Two DNA filters and one blank filter were incubated in 1 ml of the hybridization solution [30% formamide, 0.1 × SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 5

mM ethylenediaminetetraacetic acid, 10 mM Tris, *p*H 7.40] containing the ^{3}H-labeled RNA. Hybridization was carried out at 45 C for 24 hr for exhaustion hybridization. The filters were then removed from the vial, washed with 50 ml of 2 × SSC on each side, and added to a vial containing 10 μg of pancreatic ribonuclease per ml. Digestion was carried out for 30 min at 37 C. The filters were then washed and assayed for radioactivity as described previously.

Amanitine was the kind gift of T. Weiland.

RESULTS

Considerable amounts of adenovirus-specific RNA are labeled late in infection in the nuclei of the host cells. However, extensive RNA synthesis still takes place on host cell DNA in the infected cell (5). The amount of adeno-specific RNA present in the nuclei of infected cells is measured by hybridization of the RNA to an excess of adenovirus DNA bound to cellulose nitrate filters.

The RNA from nuclei of productively infected cells labeled in vivo was hybridized to adenovirus DNA filters under conditions of DNA excess. The time course of hybridization is shown in Fig. 1A. The amount of hybridization appears to plateau at approximately 14% of the input RNA, and this is independent of the amount of DNA present on the filters. It appears, therefore, that 14% of the RNA present in infected cell nuclei under the labeling conditions used is adeno-specific, and this agrees well with previous reports (5).

The RNA labeled in vitro in nuclei from infected cells was examined next. A crude nuclear preparation was incubated under the conditions described above with three unlabeled nucleoside triphosphates and radioactive uridine triphosphate (UTP). A previous report has shown that high-molecular-weight heterogeneous RNA is labeled under these conditions, although the amount of RNA elongation amounts to less than 100 nucleotides (22; L. McReynolds, *unpublished observation*). The principal activity in isolated nuclei appears to be a limited addition to preexisting molecules. The polymerase activities are inhibited by actinomycin, and the product is completely sensitive to ribonuclease.

Figure 1B shows the time course of hybridization of RNA labeled in vitro in nuclei from infected cells. The amount of material which hybridizes to adenovirus DNA appears to plateau at approximately 18% of the total labeled RNA. Thus, in RNA labeled under in vitro conditions, approximately the same fraction is adenovirus-specific as during in vivo labeling.

The activity which transcribes adenovirus DNA is apparently located in the nucleoplasm. In all the experiments described here, the nucleolar activity has been selectively suppressed by pre-

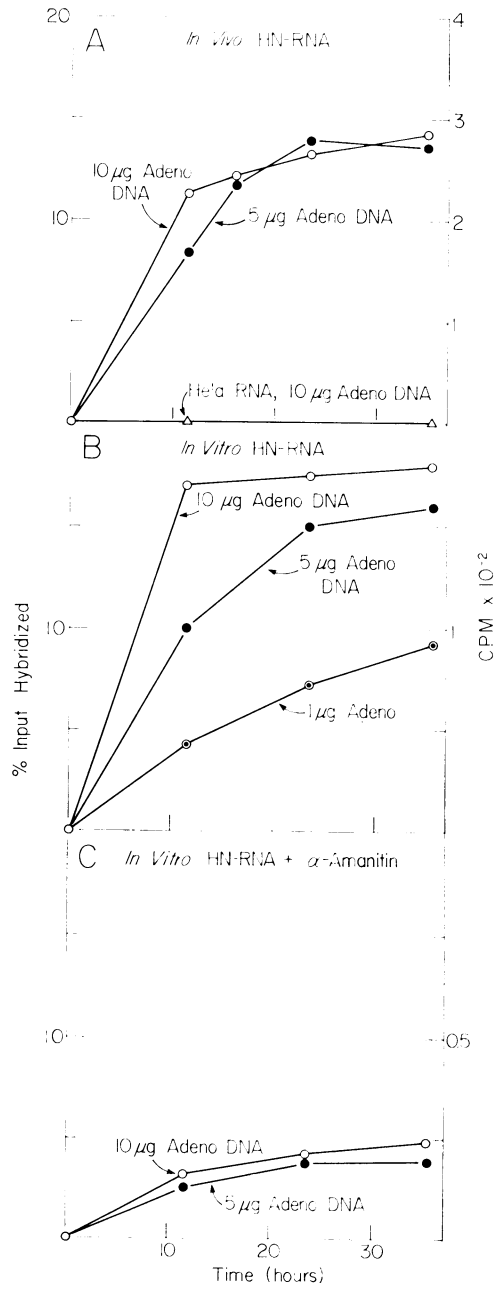

FIG. 1. *Kinetics of hybridization of nuclear RNA. A, 2 × 10⁷ HeLa cells infected with adenovirus were labeled with 200 μCi of ^{3}H-uridine for 30 min at 16 hr after infection. Nuclei were isolated and RNA was extracted as described in the text. Hybridization was as described in the text. B, Nuclei from 6 × 10⁶ infected cells at 16 hr after infection were incubated in vitro as described in the text. RNA was extracted and hybridized to adenovirus DNA as described in the text. C, Same as B except 0.2 μg of α-amanitine per ml was added to the incubation mixture.*

treating cells with low levels of actinomycin (10, 11, 15, 22).

The sensitivity of the activity which transcribes adenovirus DNA to α-amanitine was measured. This cyclic polypeptide has been shown to be a potent and selective inhibitor of polymerase II of the uninfected cell (18, 22). Nuclei from infected cells were incubated in the presence of α-amanitine. The drug suppresses in vitro incorporation to about 15% of the level obtained in the control. The small amount of labeled RNA obtained from amanitine-treated nuclei hybridizes to a limited degree with the adenovirus DNA. A plateau of approximately 6% of the input RNA labeled in the presence of α-amanitine is obtained (Fig. 1C). Thus, only a small percentage of the small amount of residual synthetic activity observed in the amanitine-treated nuclei is adenovirus-specific. Therefore, the bulk of adenovirus DNA is transcribed by an α-amanitine-sensitive polymerase. The amanitine-resistant adenovirus-specific activity produces RNA which is quite different from the nuclear heterogeneous RNA produced by the amanitine-sensitive activity. This activity will be described in detail in another report. (R. Price and S. Penman, *manuscript in preparation*).

The polymerase activities measured in vitro can be further characterized by examining their response to altered ionic strength conditions. In particular, polymerase II is very sensitive to ionic strength, in contrast to polymerase III which is comparatively indifferent to the ionic conditions of incubation (16, 22). The nucleoplasmic incorporation in incubated infected cell nuclei is shown in Fig. 2 as a function of varying concentrations of ammonium sulfate. Both total incorporation and adenovirus-specific synthesis increase significantly with increasing ammonium sulfate up to concentrations of 75 to 100 mM. Above this concentration, experimental results become variable possibly due to instability of the nuclear structure at the higher ionic strengths. The response of adenovirus-specific transcription to changes in ionic conditions resembles that of the total incorporation (which under these conditions is due principally to an activity which resembles polymerase II). It may be noted that small but reproducible differences in response to ionic strength are noted between adeno-specific transcription and total nuclear incorporation. This suggests the possibility that the polymerase activity transcribing the adenovirus genome may in fact differ from the host enzyme in some way. However, this type of experiment cannot rule out a difference in response being due to the different template used by activities transcribing the host and viral DNA.

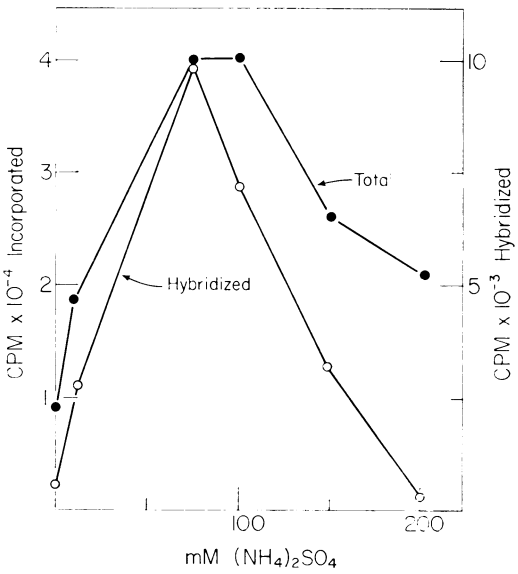

FIG. 2. *Effect of ionic strength on total and adenovirus-specific RNA synthesis. Nuclei were isolated from HeLa cells 16 hr after infection. Cells were treated with 0.04 μg of actinomycin per ml prior to fractionation for 30 min to suppress ribosomal RNA synthesis. Crude nuclei were prepared as described in the text and resuspended in the incubation medium containing the indicated concentrations of ammonium sulfate. Nuclei from 4 × 10⁶ cells were used in each sample. ³H-UTP incorporation was allowed to proceed for 5 min. Adenovirus-specific RNA synthesis was determined by hybridization as described in Fig. 1.*

The effect of manganese on the polymerase activities measured in vitro in nuclei is somewhat different than its effect on purified enzymes. In a previous report, it was shown that polymerase II activity is inhibited by manganese, whereas that resembling polymerase III was relatively indifferent to the ion. Figure 3 shows the response of total nucleoplasmic incorporation in nuclei exposed to increasing concentrations of manganese ion in the absence of magnesium. The incorporation decreases as manganese concentration increases, in agreement with previous results. The adenovirus-specific RNA, measured by hybridization, also decreases in the presence of manganese and continues to constitute a relatively constant fraction of the total nuclear RNA. The adenovirus transcription activity thus resembles host cell polymerase II by this criterion.

The product of polymerase II in uninfected cells has been shown to be primarily Hn-RNA (22). Figure 4 shows the sedimentation profile of RNA labeled by nuclei obtained from adeno-infected cells. The principal product labeled in this case is also Hn-RNA. Hybridization of fractions across

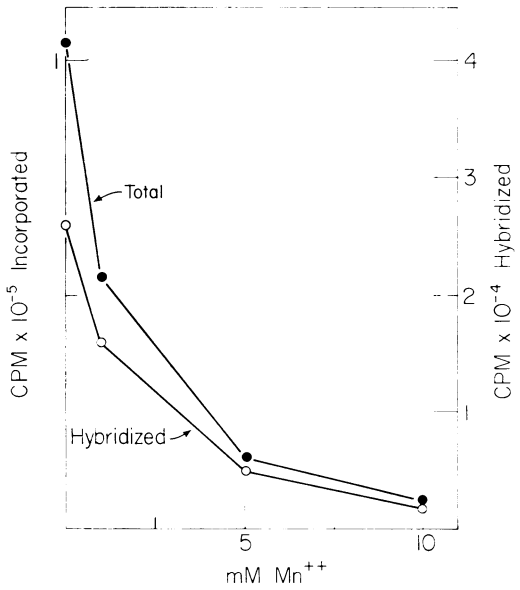

FIG. 3. *Effect of the manganese ion on total and virus-specific RNA synthesis. Nuclei were isolated from HeLa cells 16 hr after infection with adenovirus. Labeling and determination of adenovirus-specific synthesis were as described in Fig. 2.*

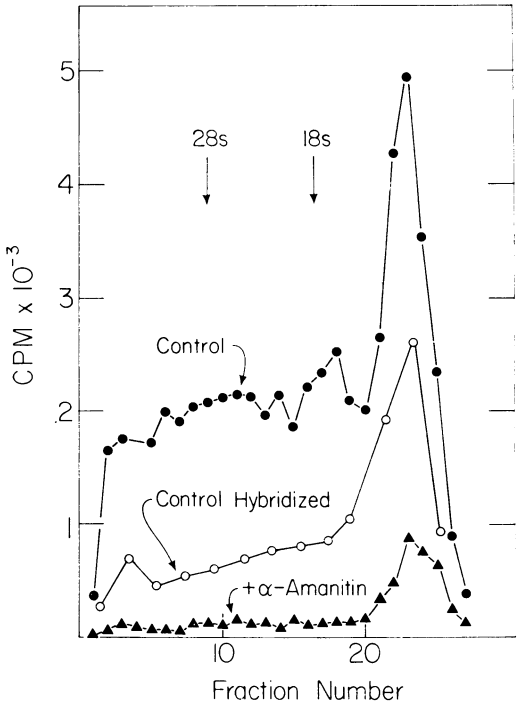

FIG. 4. *Sedimentation profile of RNA synthesized in vitro. Nuclei were isolated from 1.2 × 10⁷ HeLa cells 16 hr after infection with adenovirus and labeled with ³H-UTP as described in Fig. 2. RNA was extracted by the phenol-SDS method and analyzed by sedimentation through a 15 to 30% SDS sucrose gradient as described in the text. Centrifugation was for 16 hr at 24,000 rev/min in an SW27 rotor. Fractions were collected, and radioactivity was measured as described in the text. Portions of each fraction were precipitated with ethanol, resuspended in the hybridization solution, and hybridized to adenovirus DNA as described in the text.*

the gradient indicates that adeno-specific RNA is heterogeneous in sedimentation values and resembles Hn-RNA. The amanitine-resistant activity is also shown, and this consists primarily of low-molecular-weight RNA, which will be described in a separate report.

The adenovirus-specific RNA labeled early in infection differs in its hybridization properties from RNA produced late in infection (2, 7, 8). The properties of this early transcription were measured. Nuclei were obtained from infected cells early in the lytic cycle and incubated in vitro. Hybridization demonstrates a small amount of adeno-specific RNA synthesis, which is largely inhibited by α-amanitine. The results are summarized in Table 1. Figure 5 shows the response to ammonium sulfate of both the total incorporation and the adeno-specific transcription found in nuclei from early stages of infection. Here again the response of adeno-specific RNA labeling resembles that of the major incorporation activity of the cell, and it appears that the early RNA production in adenovirus-infected cells is also mediated through an activity resembling polymerase II.

TABLE 1. *³H-uridine triphosphate (UTP) incorporation into isolated nuclei of HeLa cells 4 hr after infection*

Test[a]	Total UTP incorporated (counts/min)	Counts/min/vial	UTP hybridized (counts/min)	Adeno-specific RNA (counts/min)
Control	126,000	24,000	320	1,675
+ α-Amanitine (0.5 μg/ml)	7,600	1,600	45	212

[a] Nuclei were isolated from cells at 4 hr after infection and incubated in vitro as in Fig. 5. The RNA was extracted and hybridized as in Fig. 1. The hybridization reaction was allowed to go to completion with excess adenovirus DNA.

DISCUSSION

Superficially, at least, the production of messenger RNA from the adenovirus genome

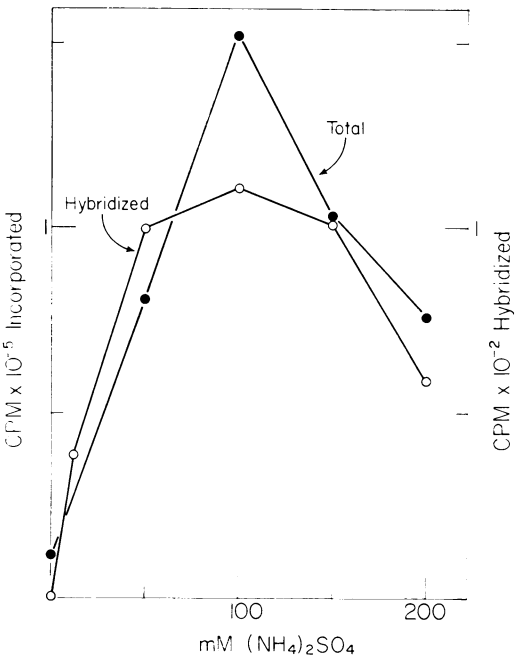

FIG. 5. *Effect of ionic strength on total and adenovirus-specific RNA synthesis. Nuclei were isolated from 2.4 × 10^7 HeLa cells 4 hr after infection. One hour after virus attachment and resuspension of the cells in fresh medium, cycloheximide was added to a concentration of 25 μg/ml to prevent the synthesis of late RNA. RNA was then analyzed by hybridization for virus-specific sequences as described in Fig. 1.*

resembles the process occurring in the host cell. Large RNA is produced in the nucleus and cleaved to smaller size, polyadenylic acid is attached, and the messenger molecules are exported to the cytoplasm (5, 12–14). However, it is not known whether the mechanisms of messenger RNA production are precisely the same as those used by the host cell. For example, it is apparent that large regions of the host cell genome are transcribed but never appear in the cytoplasm. In contrast, a major fraction of the adenovirus genome must code for the proteins known to be produced during adenovirus lytic growth. Therefore, at least part of the time, most of the RNA sequences transcribed from the adenovirus genome must appear in the cytoplasm; thus the processing of this RNA may differ in detail from that of the host cell.

This report describes the investigation of the polymerase activity which transcribes the adenovirus genome. The experiments were designed to study the polymerase activities obtained in situ in intact nuclei from infected cells. Previous experiments have shown that the RNA products obtained from HeLa cell nuclei incubated in vitro resemble those labeled in vivo. Furthermore, by several independent criteria, the properties of the polymerase activities measured in vitro resemble those of the purified enzyme preparations described by other workers. Thus, it is possible to identify the nature of the RNA product with a particular polymerase activity.

The results reported here indicate that the major portion of transcription of the adenovirus genome is accomplished by an activity resembling polymerase II. The activity is sensitive to α-amanitine and is greatly stimulated by increasing ionic strength. The polymerase activity transcribing the adenovirus genome also resembles the major nucleoplasmic activity of the host cell in its apparent inhibition by manganese ion. This is in contrast to the results reported for the purified polymerase II. However, results to be reported elsewhere will show that much of the apparent inhibition due to manganese ion results from an earlier termination of the polymerase reaction rather than from a decreased rate of RNA elongation.

The major fraction of adenovirus transcription is accomplished by polymerase activities of similar properties both early (3 to 5 hr) and late (16 to 18 hr). An examination of the ionic strength dependence of the adeno-specific transcription activity late in infection (Fig. 2) suggests that it may not be identical to the host polymerase. Possibly a polymerase modification or a subunit substitution has occurred. However, it is also possible that the small alterations in the enzyme properties may be due to its association with a template different from the normal host DNA. The experiments described here cannot distinguish between these two possibilities, and a resolution of this question probably will require detailed examination of purified enzyme preparations.

The results in Fig. 4 show that the in vitro RNA labeled by the polymerase activity transcribing the adenovirus genome resembles the RNA found in the nuclei of cells labeled in vivo. Occasionally, a pronounced peak is seen in the region of 35*S* which might correspond to one complete transcription of the adenovirus genome. However, this result has not been reproducible, and its significance is unknown. Recently Ledinko reported inhibition of adenovirus growth in human embryonic kidney cells by exposure to α-amanitine (6). Most cell lines are insensitive to the drug, probably because the polypeptide does not cross the cell membrane. However, the kidney line may retain the sensitivity that these cells show in the intact animal.

It is interesting to note the effect of adenovirus

infection upon the host polymerase activities. Considerable Hn-RHA is still transcribed from the host genome, as is evidenced in Fig. 4 and in agreement with previous reports. This RNA, however, is not successfully processed to the cytoplasm where, late in infection, only adenovirus-specific RNA appears. Thus, one result of adenovirus infection apparently is the inhibition of successful processing of Hn-RNA to messenger RNA product. Also, the results in Fig. 4 indicate an almost complete suppression of the large RNA transcribed by the amanitine-resistant activity.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA-08416-06 from the National Cancer Institute and grant GB-27684 from the National Science Foundation. One of us (S.P.) is the recipient of Public Health Service Career Development Award GM-16172-05 from the National Institute of General Medical Sciences.

The capable assistance of Deana Fowler is gratefully acknowledged.

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