State of Adenovirus 2 Deoxyribonucleic Acid in the Nucleus and Its Mode of Transcription: Studies with Isolated Viral Deoxyribonucleic Acid-Protein Complexes and Isolated Nuclei

ROBERT D. WALLACE AND JOSEPH KATES

Department of Chemistry, University of Colorado, Boulder, Colorado 80302

Received for publication 28 December 1971

Newly replicated adenovirus 2 deoxyribonucleic acid (DNA) can be isolated from the nucleus of HeLa cells by a gentle lysis procedure as a fairly homogeneous complex with a sedimentation of 73S. The viral DNA complex can be prepared completely free from host cell DNA. The viral complex is slightly active in ribonucleic acid (RNA) synthesis in vitro. Treatment of the complex with Pronase and sodium dodecyl sulfate converts the DNA to a form which sediments at 43S. Nuclei isolated from adeno-infected cells synthesize high-molecular-weight virus-specific RNA in vitro. Optimal RNA synthesis requires a divalent cation, preferentially manganese, and relatively high salt concentrations. The synthesis of virus-specific RNA by the isolated nuclei is strongly inhibited by low doses of α-amanitine. The latter experimental result is discussed in terms of the polymerase used to transcribe the adenovirus DNA in vivo.

Recent studies of viral deoxyribonucleoprotein complexes isolated from infected cells (viral DNA-protein complexes) have focused attention on these structures as a means of understanding the structural and functional organization of the viral genome within the living host cell (3, 4, 9, 10, 19). In several of the cases studied, bacteria infected with T4 (19) and lambda phages (10), or animal cells infected with vaccinia virus (3, 4), the viral DNA-protein complexes which have been isolated can support ribonucleic acid (RNA) synthesis in vitro by virtue of an endogenous RNA polymerase activity. This is also true in the case of a DNA-protein complex isolated from bacteria, consisting principally of the bacterial genome in a compact state with associated RNA polymerase and nascent RNA chains (14, 21). In general, the RNA synthesized in vitro by all of the above complexes resembles qualitatively the RNA synthesized in vivo at the time of isolation of the DNA-protein complex. In fact, it seems likely that the complexes isolated to date are only capable of completing RNA chains in vitro and not able to initiate new RNA chains.

In this report we describe a procedure for the separation of an adenovirus DNA-protein complex from the bulk of the cellular chromatin. The adenovirus DNA-protein complex was isolated by a modification of the basic procedure used by Melvin Green et al. (9) for the isolation of a DNA-protein complex from polyoma virus-infected cells. The adenovirus complex is free from host DNA and is capable of synthesizing small quantities of adenovirus RNA in vitro.

The second part of this communication describes the synthesis of adenovirus RNA by nuclei isolated from infected cells. The properties of the in vitro reaction are discussed with regard to the question of which RNA polymerase is used to transcribe the adenovirus DNA in nuclei isolated from infected cells. Although it is likely that the small nuclear viruses, polyoma and simian virus 40, must rely on the host cell RNA polymerase, the larger DNA nuclear viruses such as adenoviruses and herpes viruses may contain enough genetic information to code for their own RNA polymerases. In this study we present evidence in favor of the utilization of the host cell RNA polymerase II, thought to be located in the nucleoplasm (16), for the transcription, at least in part, of the adenovirus genome. Similar evidence supporting the use of polymerase II for adenovirus transcription has been obtained independently by Price and Penman (15).
MATERIALS AND METHODS

Cells and virus. HeLa S-3 cells were grown in Spinner culture with Eagle F 14 medium (Grand Island Biological) supplemented with 5% calf serum. Adenovirus 2, strain adenoid 6, was used throughout these studies. The virus was grown in HeLa cells and purified essentially as described by Green and Piña (8). Purified virus was stored at 1 × 10^{17} physical particles/ml in a storage buffer containing 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.1), 0.15 M NaCl, 0.1% (v/v) bovine serum albumin (crystalline, fraction V, Calbiochem), and 50% (v/v) glycerol. The virus was kept at −20 C, but the solution did not freeze. Under these conditions, no appreciable loss of infectivity was observed after a year of storage of a virus preparation.

Cells were infected at a concentration of 1 × 10^6 cells/ml after resuspending them in fresh growth medium, minus the calf serum. About 25 plaque-forming units of 500 to 1,000 physical particles of adenovirus 2 were added per cell. The adsorption period was 40 min at 37 C with stirring. The infected cells were then centrifuged, and the adsorption medium was discarded. The cells were resuspended in growth medium supplemented with 5% calf serum at a concentration of 4 × 10^6 cells/ml and were incubated at 37 C. Time zero was taken as the time of resuspension of the infected cells into the growth medium.

Isolation of nuclei. Two methods were employed for isolation of nuclei. The first method (Method I), which was used only in some of the initial experiments, was based on breakage of the cells in hypotonic buffer as previously described (11). The second method (Method II) consisted of breaking the cells by gentle pipetting at 5 C for 3 to 5 min in a buffer containing 0.01 M Tris-hydrochloride (pH 7.4), 0.6 M sucrose, 0.001 M MgCl_2, 0.005 M 2-mercaptoethanol, and 0.5% (v/v) Triton X-100. The nuclei were collected by centrifugation at 1,000 × g for 3 min and suspended at a concentration of 5 × 10^7 nuclei/ml in a buffer containing 0.05 M Tris-hydrochloride (pH 7.8), 0.004 M 2-mercaptoethanol, and 20% (v/v) glycerol. Usually, 0.05 ml of this nuclear suspension was used for assays of RNA synthesis in vitro.

Isolation of adenovirus DNA complex. The first method employed consisted of suspension of nuclei at 3 × 10^7/ml in a buffer containing 0.05 M Tris-hydrochloride (pH 8.0), 0.2 M NaCl, 0.01 M ethylenediaminetetraacetate (EDTA), and 0.25% Triton X-100 (9). Gentle pipetting of the nuclei at 0 C for 3 min was sufficient to cause lysis. The lysed nuclei, about 3 ml, were layered on a 25 to 40% (w/v) sucrose gradient dissolved in the same buffer used for lysis and centrifuged for 80 min at 10 C in the Beckman SW27 rotor at 25,000 rev/min.

Except for the experiment presented in Fig. 1, all preparations of adenovirus DNA complexes were carried out by the following method. Nuclei prepared by the Triton X-100 lysis technique in 0.6 M sucrose (see above) were suspended at 2 × 10^7/ml in 0.3 M (NH_4)_2SO_4 containing 0.004 M mercaptoethanol and 0.01 M Tris-hydrochloride (pH 7.8). The nuclei were allowed to stand at 0 C for 3 min and were then diluted twofold with 0.05 M Tris-hydrochloride (pH 7.8). After dilution, the partially lysed nuclei were layered over a cushion of 30% (w/v) sucrose in 0.05 M Tris and centrifuged for 15 min at 5 C and 20,000 × g. The liquid above the sucrose cushion was collected with a Pasteur pipet, and it contained all of the viral complex and none of the host cell nuclear DNA. This fraction was further fractionated as described in the text.

RNA synthesis by isolated nuclei. The basic reaction mixture contained in a total volume of 0.3 ml: Tris-hydrochloride (pH 7.5), 15 μmoles; (NH_4)_2SO_4, 105 μmoles; MnCl_2, 1.4 μmoles; guanosine, cytidine, and adenosine triphosphates (GRP, CTP, ATP), 0.1 μmole of each; uridine triphosphate (UTP), 0.08 μmoles and 2 μCi of ^3H-UTP (21 Ci/m mole). About 2 × 10^7 nuclei were present in this reaction mixture, and incubation was for 30 min at 37 C. If higher concentrations of nuclei were used, clumping and precipitation of the nuclei became a serious problem during the incubation. The reaction was stopped by the addition of 2 ml of 5% trichloroacetic acid. The acid-insoluble material was collected on Whatman filter papers, washed with 20 ml of cold 5% trichloroacetic acid, dried, and counted by liquid scintillation spectrometry.

Purification of nucleic acids and hybridization experiments. RNA was purified from the intravirion reaction mixtures as previously described (4). DNA was purified by the method described by Doerfler (5). DNA-RNA hybridizations were carried out by the method of Bolle et al. (2). The reactions were incubated for 8 hr at 60 C using 10 μg of heat-denatured DNA in each assay for virus-specific RNA.

Sucrose density gradient fractionation of RNA. RNA synthesized in vitro by isolated nuclei was analyzed by centrifugation through a 15 to 30% (w/v) sucrose density gradient in 0.01 M Tris-hydrochloride (pH 7.4) containing 0.01 M EDTA, 0.1 M NaCl, and 0.2% sodium dodecyl sulfate (SDS) (NETS buffer). The gradient was centrifuged for 15.5 hr at 16,000 rev/min and 25 C in a SW27 rotor of a Beckman ultracentrifuge. Before being run, the RNA sample was dissolved in 90% dimethylsulfoxide and quickly diluted with three volumes of the gradient buffer. The latter step was introduced to minimize the chances of aggregation in the RNA sample.

RESULTS

Separation of adenovirus DNA-protein complex from cellular DNA. Two methods were used initially for the isolation of the adenovirus DNA-protein complexes. Method I (see Materials and Methods) utilized nuclei prepared in hypotonic buffer, and these nuclei were lysed by resuspension in a buffer which contained 0.25% Triton X-100, 0.2 M NaCl, 0.01 M EDTA, and 0.05 M Tris-hydrochloride buffer, pH 8.0. The latter lysing procedure was suggested to us by Melvin Green of the University of California (La Jolla). To distinguish between host cell DNA and viral DNA, cells were labeled for 4 to 5 hr prior to infection with ^3H-thymidine. Prior to infection...
the cells were washed with fresh medium to remove the \textsuperscript{14}C-thymidine, and at 15 hr postinfection with adenovirus the cells were labeled with \textsuperscript{3}H-thymidine for 2 hr in the presence of \textsuperscript{10} C fluorodeoxyuridine which enhanced incorporation of radioactive thymidine into viral DNA. The time of labeling of viral DNA with \textsuperscript{3}H-thymidine was chosen on the basis of prior results which showed that host DNA synthesis is almost completely inhibited at 15 hr postinfection, while virus DNA synthesis occurs at an appreciable rate (7). The doubly labeled nuclear lysate was layered over a 25 to 40\% (w/v) sucrose density gradient which was centrifuged for 80 min at 10 C at 25,000 rev/min in the SW27 rotor of the Spinco ultracentrifuge. Figure 1 illustrates the results obtained in the latter experiment, showing a complete separation of the viral DNA complex from the cellular DNA. The viral DNA counts are found in a rather homogeneous peak in the upper half of the gradient, whereas all of the host DNA is present in the pellet.

A second method (Method II, see Materials and Methods) for preparation of nuclei and adenovirus DNA-protein complexes was developed and used for all subsequent experiments presented in this paper. Method II was preferred because nuclei prepared by this procedure were more active in RNA synthesis in vitro and because isolation of the adenovirus DNA complex was somewhat more convenient. After preparation by Method II, the viral DNA-protein complex was further purified by sedimentation in a 10 to 30\% (w/v) sucrose density gradient. The complex sedimented as a fairly homogeneous component with a mean sedimentation coefficient of approximately 74S (Fig. 2). This sedimentation value is considerably higher than mature adenovirus DNA (32S). Whole adenovirus virions sediment to the bottom of the gradients employed to band the complex.

Effects of treatment of the DNA complex with Pronase and SDS. When the adenovirus DNA-protein complex was treated for 30 min with 1 mg of heat-treated Pronase per ml and then with 0.2\% SDS at 37 C for an additional 30 min, the DNA sedimented in a sucrose gradient with a sedimentation of about 42 to 45S which is appreciably faster than mature viral DNA (Fig. 3).

Synthesis of RNA in vitro by isolated complex. The ability of the viral DNA complex to synthesize RNA was tested by assaying each fraction of a sucrose gradient in which the complex had sedimented as a band for ability to synthesize RNA from added nucleoside triphosphates. Figure 4 shows a fair degree of correspondence between ability to synthesize RNA and the counts-per-minute profile of the DNA complex in the gradient. It should be pointed out that at the peak fraction 6.8 pmoles of uridine monophosphate (UMP) was incorporated into RNA in 30 min when 2.5 \mu g of DNA, as DNA complex, was added to the assay mixture. This represents a low level of RNA synthesis associated with the complex. It should also be noted that some synthesis of RNA was observed consistently in areas of the gradient which contained little viral DNA. In view of the poor incorporation of precursors into RNA by the complexes, further studies of viral RNA synthesis in vitro were carried out with isolated nuclei.

Synthesis of RNA by isolated nuclei. Synthesis of RNA in vitro by nuclei isolated from infected cells at 15 hr postinfection possesses the characteristics of a DNA-dependent RNA polymerase reaction (Table 1). Synthesis requires a divalent cation, is inhibited by treatment of the nuclei with deoxyribonuclease or actinomycin D, and is partially dependent on the addition of exogenous...
nucleoside triphosphates. The requirement for exogenous ATP is only partial, indicating that the isolated nuclei may possess a pool of this substrate. The product synthesized appears to be RNA since it is completely sensitive to digestion by pancreatic ribonuclease.

The nuclei isolated from infected cells can utilize either MnCl₂ or MgCl₂. The optimal MnCl₂ concentration is 5 mM, and the optimal MgCl₂ concentration is 7.5 mM. The activity with MnCl₂ is generally somewhat higher than with MgCl₂, but the difference in activity is less than twofold. The reaction in the presence of MnCl₂ with both infected and uninfected nuclei is enhanced by the presence of rather high concentrations of (NH₄)₂SO₄ (Fig. 5). The enhancement of activity is more pronounced for adeno-infected nuclei than for uninfected nuclei.

Figure 6 demonstrates the time course of RNA synthesis by isolated nuclei from infected and uninfected cells. Although the initial rate of synthesis appears roughly comparable in both types of nuclei, the nuclei from infected cells continue to synthesize RNA for longer periods of time. The latter observation may account for the fact that nuclei from adeno-infected cells prepared at different times after infection show considerably more activity in vitro in a 30-min assay than uninfected nuclei. Figure 7 demonstrates the ability of nuclei isolated from infected cells to synthesize RNA in vitro as a function of time after infection. The data are presented relative to the amount of RNA synthesized by nuclei isolated from uninfected cells. A maximum in the ability to synthesize RNA in vitro is reached with nuclei isolated at about 15 hr postinfection. At this time, nuclei isolated from infected cells are about 4.5 times more active than uninfected cell nuclei.

The fact that the RNA synthesized in vitro by nuclei from adeno-infected cells represents a high proportion of RNA coded from the viral genome may be deduced from the hybridization experiment shown in Table 2. More than 30% of the RNA synthesized in vitro by nuclei isolated at

![Figure 2](http://jvi.asm.org/)

**Fig. 2.** Sedimentation of adenovirus DNA complex in a sucrose density gradient. Adenovirus DNA complex prepared from nuclei lysed in the presence of 0.3 M (NH₄)₂SO₄ (see Materials and Methods) was centrifuged through a 10 to 30% (w/v) sucrose gradient in a Beckman SW27 rotor at 25,000 rev/min at 10 C. Ribosomal monomers from HeLa cells were included in the gradient as sedimentation markers. Sucrose was dissolved in 0.05 M Tris-hydrochloride, pH 8.0.

![Figure 3](http://jvi.asm.org/)

**Fig. 3.** Sedimentation of the adenovirus DNA complex after treatment with proteolytic enzyme and sodium dodecyl sulfate (SDS). Viral DNA complex prepared as described in Materials and Methods was treated with 0.8 mg of heat-treated Pronase per ml (4) at 37 C for 30 min. SDS was added to a final concentration of 0.2% (w/v), and incubation was continued at 37 C for 30 min. The sample was then centrifuged in a sucrose density gradient, 10 to 30% (w/v) in 0.5 M Tris (pH 8.0), for 8 hr at 27,000 rev/min and 10 C in a Beckman SW27 rotor. Fifty micrograms of mature, intact adenovirus DNA was used as a sedimentation marker. Counts per minute in DNA from complex (○); optical density at 260 nm of mature adenovirus DNA (●).
FIG. 4. RNA synthesis by adenovirus DNA complex. The DNA complex was prepared and centrifuged as described in legend of Fig. 2. A portion of each fraction from the gradient was assayed for ability to synthesize RNA in vitro. Assay conditions used were similar to those described for isolated nuclei (see Materials and Methods). $^{14}$C-thymidine counts/min representing the complex DNA (○); $^{3}H$-UMP incorporated into RNA (□).

Table 1. RNA synthesis in vitro by nuclei isolated from adenovirus 2-infected HeLa cells

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Picomoles of $^{3}H$-UMP incorporated</th>
<th>Per cent activity of complete system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>384</td>
<td>100</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>174</td>
<td>46</td>
</tr>
<tr>
<td>Minus GTP</td>
<td>49</td>
<td>12</td>
</tr>
<tr>
<td>Minus CTP</td>
<td>77</td>
<td>18</td>
</tr>
<tr>
<td>Minus Mn$^{2+}$</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Minus Mn, plus Mg$^{2+}$</td>
<td>56</td>
<td>15</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>219</td>
<td>57</td>
</tr>
</tbody>
</table>

several times after infection hybridizes with adenovirus DNA. This estimate of the fraction of RNA which is adenovirus-specific is likely to be low due to possible inefficiency associated with the hybridization experiment.

FIG. 5. Effect of ammonium sulfate on the synthesis of RNA by isolated nuclei. Nuclei from infected cells were prepared at 15 hr postinfection. Assay mixture contained in a volume of 0.3 ml: Tris-hydrochloride (pH 7.5), 15 μmoles; (NH₄)₂SO₄, 105 μmoles; MnCl₂, 1.5 μmoles; GTP, ATP, CTP, 0.1 μmoles of each; UTP, 0.08 μmoles; and 2 μCi of $^{3}H$-UTP. Each assay contained 2 × 10⁶ nuclei. The reaction mixture was made up to the desired salt concentration before addition of the nuclei. Nuclei from infected cells (●); nuclei from uninfected cells (○).

FIG. 6. Time course of RNA synthesis in vitro by isolated nuclei from infected and uninfected cells. Nuclei from infected cells were prepared at 18 hr postinfection. Assay conditions were the same as described in legend of Fig. 5. Nuclei from infected cells (●); nuclei from uninfected cells (○).
The size distribution of the RNA synthesized by nuclei isolated from adenovirus-infected cells at 15 hr postinfection is shown in Fig. 8. It is clear that some species of RNA with sedimentation coefficients greater than 28S are synthesized. In another experiment, RNA taken from a similar gradient representing fractions greater than 45S in size was found to hybridize with adenovirus DNA to an extent comparable to total RNA, before sucrose fractionation (unpublished data). This indicates that adenospecific RNA sequences synthesized in vitro occur in very large molecules resembling nucleoplasmatic heterogeneous nuclear RNA (Hn RNA) from uninfected cells.

**Effect of α-amanitine on RNA synthesis by isolated nuclei.** The drug α-amanitine (6, 20, 24) has been demonstrated to be a powerful inhibitor of the polymerase II present in the nucleoplasm of mammalian cells (12). Addition of the drug to isolated nuclei has been shown to inhibit the synthesis of Hn RNA in the case of uninfected HeLa cells, but not to inhibit the synthesis of ribosomal RNA precursors (25), which are synthesized in the nucleolus, presumably by RNA polymerase I. In addition to RNA polymerase II there is another enzyme, RNA polymerase III, which is thought to be present in the nucleoplasm and which may also synthesize large Hn RNA (15, 16, 25). RNA polymerase III is not inhibited by α-amanitine (12). In view of the high specificity of α-amanitine inhibition of polymerase II, we decided to utilize this drug to determine whether RNA polymerase II was involved in the tran-

![Fig. 7. RNA synthesis by nuclei isolated from adenovirus-infected cells as a function of time after infection. Assay conditions were the same as described in legend of Fig. 3 and incubation was for 30 min at 37 C. Activity of the infected nuclei is expressed as a factor of the activity present in uninfected nuclei.](http://jvi.asm.org/)

**TABLE 2. DNA-RNA hybridization of RNA made in vitro at different times after infection**

<table>
<thead>
<tr>
<th>In vitro RNA prepared from nuclei</th>
<th>Per cent hybridized to adenovirus 2 DNAa</th>
<th>Per cent hybridized to HeLa DNAa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>4.4 (0.6)b</td>
<td>3.8 (3.5)b</td>
</tr>
<tr>
<td>10 hr PIc</td>
<td>22.5</td>
<td>0.7</td>
</tr>
<tr>
<td>12 hr PIc</td>
<td>34.7 (10 µg of DNA)</td>
<td>0.9</td>
</tr>
<tr>
<td>12 hr PIc</td>
<td>34.3 (20 µg of DNA)</td>
<td></td>
</tr>
<tr>
<td>16 hr PIc</td>
<td>28.8</td>
<td>1.3</td>
</tr>
<tr>
<td>20 hr PIc</td>
<td>18.3</td>
<td>1.2</td>
</tr>
<tr>
<td>22 hr PIc</td>
<td>26</td>
<td>1.3</td>
</tr>
</tbody>
</table>

a Unless otherwise noted, 10 µg of denatured DNA was used. Values represent average of duplicate points obtained in same experiment. b Values obtained in a different experiment. c PI = postinfection.

![Fig. 8. Size distribution in a sucrose density gradient of RNA synthesized in vitro by nuclei isolated from adenovirus-infected cells. The nuclei used in the in vitro reaction were isolated at 15 hr postinfection. RNA was purified as described in Materials and Methods and then centrifuged through a 15 to 30% (w/v) sucrose density gradient made in NETS buffer for 15.5 hr at 16,000 rev/min and 25 C in a Beckman SW27 rotor. Fractions were collected from the bottom of the tube.](http://jvi.asm.org/)
scription of the adenovirus genome in vitro. A similar experiment could not be carried out in vivo because α-amanitine is not taken up appreciably by living HeLa cells.

It may be seen in Fig. 9 that α-amanitine at very low concentrations inhibited by 80 and 70% the amount of RNA synthesized in vitro by nuclei isolated from infected and uninfected cells, respectively. This means that a majority of the RNA synthesized in the presence of Mn²⁺ and high (NH₄)₂SO₄ was inhibited by the drug. The amount of residual RNA synthesis observed in the presence of the drug could be accounted for by slight continued transcription by the nucleolar polymerase and by polymerase III of the nucleoplasm, which are not sensitive to the drug. The reduced amount of RNA synthesized in the presence of α-amanitine by infected nuclei was tested for its ability to hybridize with adenovirus DNA. As shown in Table 3, there was a lower proportion of virus-specific RNA in the α-amanitine-resistant fraction, than in the total RNA synthesized by uninfected nuclei from infected cells. Thus, inhibition by α-amanitine of virus-specific RNA closely resembles inhibition of uninfected-cell nuclear RNA synthesis. This is taken as evidence that polymerase II may be utilized, at least in part, to transcribe the adenovirus DNA in nuclei prepared from infected cells.

![Figure 9](image)

**FIG. 9. Effect of α-amanitine on RNA synthesis in vitro by nuclei from infected and uninfected cells. Reaction conditions were the same as described in Fig. 5. α-Amanitine was added before addition of nuclei to reaction mixture. RNA synthesis by nuclei from infected cells (○); and uninfected cells (●).**

<table>
<thead>
<tr>
<th>Nuclei in reaction†</th>
<th>Incorporation into RNA</th>
<th>Per cent input RNA hybridized with adenovirus 2 DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>8,560</td>
<td>100</td>
</tr>
<tr>
<td>Infected plus α-amanitine</td>
<td>1,650</td>
<td>19</td>
</tr>
</tbody>
</table>

† Nuclei were prepared at 15 hr postinfection. Ten micrograms of adenovirus 2 DNA were used for the hybridization experiments.

**DISCUSSION**

Newly replicated adenovirus DNA can be readily isolated free from host cell DNA, as a complex with a sedimentation coefficient of approximately 73S. Treatment of this complex with Pronase and SDS reduces the sedimentation of the DNA to about 43S. This is considerably higher than 32S, the sedimentation coefficient of mature adenovirus 2 DNA (5). The 43S sedimentation coefficient may result from the fact that this DNA is twice as long as the mature viral DNA, or, more likely, that this DNA is still associated with some other molecular species resulting in a higher particle weight and more compact structure. Perhaps protein corresponding to one or more of the internal proteins found in the adenovirus virion (18) are present in this complex. In fact, the present DNA complex may resemble, in part, the DNA-protein structure seen in the electron microscope after disruption of the adenovirus virion with SDS (23). It is likely, based on this study, that the 73S complex contains DNA-dependent RNA polymerase and nascent RNA chains in addition to any other structural proteins which might be present. It is clear that further studies of the constituents of this complex must be carried out in order to obtain a better understanding of its composition and structure. A major problem which must be confronted is the possibility that the complex is formed as a result of the isolation procedures, by adventitious association of host or viral proteins to the viral DNA. Careful reconstruction experiments with exogenously added viral DNA and kinetic studies of the entry of newly synthesized viral DNA into the complex may be useful in eliminating the latter possibility.

RNA synthesis in vitro by nuclei isolated from infected cells resembles, in some respects, the synthesis of RNA in vivo. When nuclei were isolated at different times after adenovirus infection and tested for ability to synthesize RNA
in vitro, the rates observed possessed a maximum at 15 hr postinfection. This maximal rate of synthesis was 4.5 times higher than for uninfected cell nuclei during the 30-min in vitro incubation period. RNA synthesis in vivo in adenovirus-infected cells also shows a maximum in the middle of the virus growth cycle (7). This maximum observed both in vivo and in vitro may result from the availability of viral progeny DNA template which might be the limiting factor in RNA synthesis. The decline in RNA synthesis observed at later times after infection might be due to packaging of the DNA into virions, or cytopathic effects leading to degeneration of the nuclear transcription system.

A substantial fraction of the virus-specific RNA molecules labeled in vitro are extremely large, resembling viral RNA synthesized in vivo in the nucleoplasm (13). Adenovirus RNA isolated from polyribosomes, on the other hand, is much smaller (13). It is likely that the large nuclear adenovirus RNA represents transcripts of large segments of the viral genome and is therefore likely to consist of highly polycistronic RNA. In the present study, the question of breakdown of the RNA labeled in vitro and its release from the nuclei was not studied in any detail. It was noted, however, that little, if any, breakdown of the large species of RNA occurred upon incubation at 37 C for 30 min after the reaction of RNA synthesis had gone to completion (unpublished data). The termination of RNA synthesis in isolated nuclei after about 30 min could not be reversed by the addition of fresh substrates. It might be of interest to investigate the addition of cytoplasmic or nuclear factors for their ability to restore RNA synthesis in the spent nuclei.

The fact that the isolated nuclei from infected cells were most active in the presence of Mn²⁺ and high salt [0.3 M (NH₄)₂SO₄] indicated that an RNA polymerase activity similar to that observed in the nucleoplasm and different from that observed in the nucleolus (23, 25) was used in the transcription of adenovirus DNA. Roeder and Rutter (15, 17) have characterized three RNA polymerase species in the nuclei of mammalian cells. Polymerase I is present in the nucleolus. Polymerase II and III are found in the nucleoplasm. Polymerase II, the major nucleoplasmic species, is completely inhibited by low concentrations of the drug a-amanitine (12), whereas polymerase I and III are not sensitive to the drug. It appears likely from recent studies that polymerase II is involved in the synthesis of Hn RNA in mammalian nuclei (25) and RNA polymerase III is also involved in the synthesis of a minor fraction of the Hn RNA. Our results that adeno-
specific RNA synthesis is strongly inhibited in isolated nuclei by a-amanitine suggest that at least the a-amanitine-sensitive moiety of polymerase II might be used to transcribe the adenovirus DNA in this system. This in turn suggests that polymerase II is used to transcribe adenovirus genes in vivo. Alternative explanations for the a-amanitine sensitivity of adenovirus RNA synthesis in vitro might be that adenovirus codes for a new RNA polymerase which also happens to be sensitive to the drug, or that the in vitro system is not transcribed by the same polymerase as used in vivo. However, it seems plausible that a major part of the in vitro synthesis is due to completion of RNA chains initiated in vivo. Thus it is reasoned that an a-amanitine-sensitive polymerase is actually used in vivo to transcribe the viral genome. If the virus does, in fact, use the host cell nucleoplasmic RNA polymerases, it is possible that the virus codes for factors which alter the specificity of the host enzyme(s) so as to transcribe preferentially the viral DNA or selected portions thereof.

ACKNOWLEDGMENTS

The present study was supported by Public Health Service grant ROI AI08413 from the National Institute of Allergy and Infectious Diseases.

We are grateful to J. Beeson for his valuable assistance. We thank Melvin Green of the University of California in San Diego for his advice concerning the methods of isolation of nuclear-virus DNA complexes.

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